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(54) **CELL CULTURE METHODS**

(57) The present invention relates to methods for reducing the heterogeneity of a population of recombinant proteins produced in cell culture, said methods comprising growing host cells producing a recombinant protein in a cell culture medium wherein the cell culture medium comprises one or more cysteine/cystine analogs.

EP 3 492 582 A1

Description

FIELD OF THE INVENTION

[0001] The present invention belongs to the field of the manufacture of recombinant proteins, in particular antibodies. More specifically, it relates to cell culture methods for producing recombinant proteins with reduced heterogeneity during commercial scale manufacturing.

BACKGROUND OF THE INVENTION

[0002] Development of recombinant proteins as therapeutic proteins, such as therapeutic antibodies, requires production of the recombinant proteins at an industrial scale. In order to achieve this, different expression systems, both prokaryotic and eukaryotic systems, may be employed. Over the past two decades, however, the majority of the therapeutic proteins approved as therapeutic have been manufactured through mammalian cell cultures and such systems remain the preferred expression systems for producing large quantity of recombinant proteins for human use.

[0003] Mammalian cell cultures, however, present significant challenges. The titer of recombinant protein produced is generally very low compared with other eukaryotic productions systems, such as those based on yeast and insect cells. Over the last 30 years, much effort has been dedicated to establishing the basic parameters of cell culture and recombinant protein expression with much focus of the research dedicated to reaching optimal cell growth through changes of the composition of the cell culture media (see e.g. Hecklau C., et al. J Biotech 218 (2016) 53-63; Zang Li. et al. Anal. Chem 83 (2011) 5422-5430) and operating conditions and, development of large bioreactors. For example, L-cysteine is one of the essential amino acids that is commonly added in media and feeds. Cysteine derivatives, such as S-Sulfocysteine and N-acetyl-cysteine, have been used to improve specific productivity in cell culture (Hecklau et al., supra; Oh et al. (2005) Biotechnol. Prog. 21:1154).

[0004] Whilst yield is still a very important aspect of mammalian cell culture, in recent years, the focus has shifted towards controlling product quality and process consistency at all stages of development and production scale. Therapeutic proteins produced by mammalian cell culture exhibit varying levels of heterogeneity. Such heterogeneity includes, but is not limited to, different glycosylation patterns, differences resulting from deamidation or oxidation, different charge or size variants. Heterogeneity of recombinant proteins may also lead to differences in product color, e.g. between different batches of the same protein manufactured by the same manufacturing process. Such heterogeneity and in particular differences in color, of the recombinant protein of interest, becomes more apparent when the therapeutic proteins are formulated at high concentrations. In recent years, there has been a steady trend toward subcutaneous delivery of therapeutic proteins which requires formulating therapeutic proteins at high concentrations. High concentrations have also been associated with increased aggregate levels (Purdie J., et al. Biotechnology Progress, 2016). Increased charge variants, such as increased levels of acidic species may affect protein stability (Banks D. D., et al. Journal of pharmaceutical sciences, 2009) whilst the color of the concentrated therapeutic protein may be more intense.

[0005] Cell culture conditions, such as the composition of the medium (Kshirsagar R., et al. Biotechnology and Bioengineering, 109:10, 2523-2532 (2012); US 2013/0281355; WO 2013/158275) and the growing conditions, including pH and temperature (US 8,765,413) have been shown to impact the quality attributes of therapeutic proteins. Yet, there remains the need to provide further improved cell culture methods for the production of therapeutic proteins, and in particular, therapeutic antibodies with minimal heterogeneity.

SUMMARY OF THE INVENTION

[0006] The present invention addresses the above-identified need by adding cysteine/cystine analogs and/or partially replacing cysteine/cystine by cysteine/cystine analogs in the medium used for production of recombinant proteins in cell culture.

[0007] Accordingly, in a first aspect, the invention relates to a method for reducing the heterogeneity of a population of recombinant proteins produced in cell culture, said method comprising growing host cells producing a recombinant protein in a cell culture medium wherein the cell culture medium comprises one or more cysteine/cystine analogs.

[0008] In a second aspect, the invention relates to a method for producing a recombinant protein preparation comprising:

(i) inoculating said host cells in a basal medium, wherein the basal medium optionally comprises an initial amount of:

(a) cysteine/cystine analogs; and/or

(b) cysteine and/or cystine,

(ii) progressing the culture through a production phase wherein the recombinant protein is produced by the cells, wherein, during said production phase, the cell culture medium is supplemented with:

(a) cysteine/cystine analogs; and/or

(b) cysteine and/or cystine,

wherein (a) and (b) may be added simultaneously or sequentially,
wherein, when the contents of the basal medium and the total supplements added are added up, the molar ratio of (a) to (b) is between 1:18 and 18:1.

[0009] In a further aspect, the invention relates to a recombinant protein preparation obtainable or obtained by the method according to the invention.

[0010] In an even further aspect, the invention relates to a cell culture medium suitable for culturing mammalian cells comprising N,N'-diacetyl-L-cystine-dimethylester.

BRIEF DESCRIPTION OF THE DRAWINGS

[0011]

Figure 1: Viable cell concentrations
Figure 2: Relative % change in Mab titer on day 14 compared to control (100% Cysteine Feed)
Figure 3: Relative % change in color intensity (b*value) level on day 14 compared to control (100% Cysteine Feed)
Figure 4: Relative % change in acidic species level on day 14 compared to control (100% Cysteine Feed)
Figure 5: Relative % change in main charge species level on day 14 compared to control (100% Cysteine Feed)
Figure 6: Viable cell concentrations
Figure 7: Relative % change in Mab titer on day 14 compared to control (100% Cysteine Feed)
Figure 8: Relative % change in acidic species level on day 14 compared to control (100% Cysteine Feed)
Figure 9: Relative % change in main charge species level on day 14 compared to control (100% Cysteine Feed)
Figure 10: Cell growth profile for cell line 1 in 2L bioreactor (viable cell concentrations)
Figure 11: Relative % change in Mab titer compared to control for cell line 1 in 2L bioreactor
Figure 12 Relative % change in acidic species level compared to control for cell line 1 in 2L bioreactor
Figure 13: Relative % change in main charge species level compared to control for cell line 1 in 2L bioreactor
Figure 14: Relative % change in color intensity (b* value) with respect to the control for cell line 1 in 2L bioreactor
Figure 15: Cell growth profile for cell line 2 in 2L bioreactor (viable cell concentrations)
Figure 16: Relative % change in Mab titer compared to control for cell line 2 in 2L bioreactor
Figure 17 Relative % change in acidic species level compared to control for cell line 2 in 2L bioreactor
Figure 18: Relative % change in main charge species level compared to control for cell line 2 in 2L bioreactor
Figure 19: Average cell growth profile from data sets 1 and 2 in 2L bioreactors (error bars = 1SD)
Figure 20: Relative % change in Mab titer with respect to the control. Data sets 1 and 2 average values (error bars = 1SD)
Figure 21: Relative % change in acidic species level with respect to the control. Data sets 1 and 2 average values (error bars = 1SD)
Figure 22: Relative % change in main charge species level with respect to the control. Data sets 1 and 2 average values (error bars = 1SD)
Figure 23: Relative % change in color intensity (b* value) with respect to the control. Data sets 1 and 2 average values (error bars = 1SD)

DETAILED DESCRIPTION OF THE INVENTION

[0012] As described above, in a first aspect, the invention relates to a method for reducing the heterogeneity of a population of recombinant proteins produced in cell culture, said method comprising growing host cells producing a recombinant protein in a cell culture medium wherein the cell culture medium comprises one or more cysteine/cystine analogs.

[0013] The method of the invention particularly reduces coloration of recombinant proteins. Thus, in an independent aspect, the invention relates to a method for reducing coloration of a population of recombinant proteins produced in cell culture, said method comprising growing host cells producing a recombinant protein in a cell culture medium comprising one or more cysteine/cystine analogs.

[0014] In a further independent aspect, the invention relates to a method for producing a recombinant protein prepa-

ration comprising:

(i) inoculating said host cells in a basal medium, wherein the basal medium optionally comprises an initial amount of:

(a) cysteine/cystine analogs; and/or

(b) cysteine and/or cystine,

(ii) progressing the culture through a production phase wherein the recombinant protein is produced by the cells, wherein, during said production phase, the cell culture medium is supplemented with:

(a) cysteine/cystine analogs; and/or
(b) cysteine and/or cystine,

wherein (a) and (b) may be added simultaneously or sequentially,
wherein, when the contents of the basal medium and the total supplements added are added up, the molar ratio of (a) to (b) is between 1:18 and 18:1.

Heterogeneity

[0015] The invention is based on the finding that by adding cysteine/cystine analogs to the cell culture medium during the production phase in a process for manufacturing a recombinant protein, the heterogeneity of the recombinant polypeptides produced is reduced, without reducing the titer of the recombinant polypeptides at the end of the production.

[0016] The heterogeneity is preferably reduced with respect to:

a. color or intensity of color;
b. charge heterogeneity, preferably by reducing acidic peak group species (APG) and/or basic peak group species (BPG), whereby the main charge species does not decrease; and/or
c. amino acid oxidation, preferably methionine oxidation.

[0017] The term "heterogeneity" as used herein refers to differences between individual molecules, e.g. recombinant proteins, in a population of molecules produced by the same manufacturing process, or within the same manufacturing batch. Heterogeneity can result from incomplete or inhomogeneous modifications of the recombinant polypeptides, e.g. due to post-translational modifications of the polypeptide or to misincorporation during transcription or translation. Post-translational modifications can e.g. be the result of deamination reactions and/or oxidation reactions and/or covalent addition of small molecules such as glycation reactions and/or isomerization reactions and/or fragmentation reactions and/or other reactions and also include variation on the glycation patterns. The chemo-physical manifestation of such heterogeneity leads to various characteristics in the resulting recombinant polypeptide preparations which include, but are not limited to, charge variant profile, color or color intensity and molecular weight profile.

[0018] The terms "colored" or "color" when used herein indicate that a liquid solution, such as a concentrated protein preparation is not colorless. Following the definition of the European pharmacopeia, a solution is colorless if it has the appearance of water R or the solvent or is not more intensely colored than reference solution B9 (European pharmacopeia 2.2.2). A possible way to measure the reduction of color or intensity of color of recombinant proteins in cell culture, which can be used according to this invention, is by measuring relative spectral power distribution of CIE Standard Illuminant A Color intensity using a spectrophotometer by transmission, e.g. using UltrascanPro, and by comparing the data to the CIE (commission internationale de l'éclairage) scale for example by comparing the b*value.

[0019] The reduction of the charge heterogeneity, is preferably defined by measuring the acidic peak group (APG) species in the population of recombinant proteins produced in the cell culture. A possible way to measure the APG reduction, is by determining vialmaged Capillary Electrophoresis (e.g. ProteinSimple iCE3) the relative percentage of acidic (APG for Acidic Peak Group) isoforms of the recombinant proteins produced in a cell culture medium with or without the cysteine/cysteine analogs, which recombinant protein is at time of measurement preferably purified. When measuring the isoforms of the recombination proteins, besides the APG also the basic isoforms (Basic Peak Group (BPG)) and the main charge species are measured, wherein the main charge species represents the isoform of the recombinant protein that one wishes to obtain. It is preferred that when the APG is decreased, there is substantially no increase of the BPG. Preferably, when the APG is decreased, the main charge species level increases.

[0020] As described, the invention is related to manufacturing a recombinant protein wherein the heterogeneity of the recombinant polypeptides produced is reduced, without reducing the yield of the recombinant polypeptides. Preferably, the titer of the recombinant polypeptides is increased. According to the invention "the titer" is the concentration of the

recombinant polypeptide at the end of the production phase, unless indicated differently.

Cysteine/cystine analogs

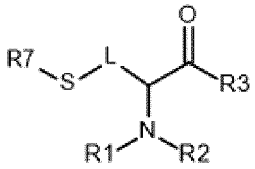
[0021] The term "cysteine analogs" or "cysteine derivatives" when used herein means one or more compounds which are structural analogs of cysteine, with the exception of cysteine itself.

[0022] The term "cystine analogs" or "cystine derivatives" when used herein means one or more compounds which are structural analogs of cysteine, with the exception of cystine itself.

[0023] The term "cysteine/cystine analogs" means "cysteine analogs" or "cystine analogs" or a mixture of "cysteine analogs" and "cystine analogs".

[0024] The term "cysteine/cystine derivatives" means "cysteine derivatives" or "cystine derivatives" or a mixture of "cysteine derivatives" and "cystine derivatives".

[0025] In one embodiment according to the present invention, the cysteine/cystine analogs comprise or consist of one or more compounds selected from the compounds represented by formula 1 and 2, and salts thereof:



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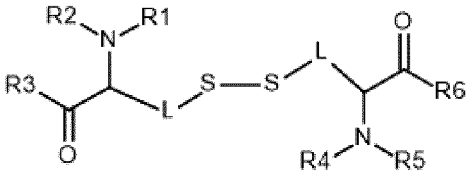

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wherein,

R1, R2, R4 and R5 independently represent hydrogen, amino carbonyl, C_{2-22}acyl, C_{1-22}alkyl which group is optionally substituted by one or two substituents selected from hydroxy and C_{1-22}alkoxy; C_{1-22} heteroalkyl, hydroxysulphonyl, C_{1-22}alkylsulphonyl, or C_{5-22}aryl sulphonyl;

R3 and R6 independently represent hydroxy; NH_2; C_{1-22}alkoxy wherein one or more carbons of the C_{1-22} alkyl may be optionally replaced by an aryl or an heteroaryl; C_{1-22} alkylamino which group is optionally substituted by a hydroxy or a C_{1-22} alkyl; hydroxy amino; C_{1-22} alkoxy amino; C_{1-22} alkylamino wherein one or more carbons of the C_{1-22}alkyl are replaced by an aryl or an heteroaryl; C_{1-22}heteroalkylamino; di(C_{1-22} alkyl)amino which is optionally substituted by a hydroxy; di(C_{1-22}alkyl)amino wherein one or more carbons of the C_{1-22}alkyl are replaced by an aryl or heteroaryl; or di(C_{1-22}heteroalkyl)amino;

R7 represents hydrogen, phosphate or sulphate.

L represents an optionally substituted C_{1-10}alkylene chain; and

with the proviso that the compound is not cysteine or cystine.

[0026] The term " C_{1-22}alkyl" as used herein refers to aliphatic hydrocarbon groups which may be straight or branched and may comprise 1 to 22 carbon atoms in the chain. Generally, C_{122}alkyl groups which may be present on the compounds of use in the invention include C_{6-22} alkyl groups, C_{12-22} alkyl groups, C_{1-16} alkyl groups, C_{1-10} alkyl groups and C_{1-6} alkyl groups. Examples of C_{12-22} alkyl groups include palmitinyl and stearyl.

[0027] The term "C_{5-22}aryl" as used herein, refers to an unsaturated aromatic carbocyclic group of from 5 to 22 carbon atoms having a single ring or multiple condensed rings. Generally, C_{5-22} aryl groups which may be present on the compounds of use in the invention include C_{5-14} aryl groups, suitably include C_{5-10} aryl groups. Examples of C_{5-22}aryl groups are phenyl and naphtyl.

[0028] The term "C_{5-22}heteroaryl" as used herein represents aromatic carbocyclic groups of from 5 to 22 carbon atoms having a single ring or multiple condensed rings, wherein one or more of the said carbon atoms have been replaced by one or more heteroatoms selected from oxygen, sulphur and nitrogen. Generally, C_{5-22} heteroaryl groups which may be present on the compounds of use in the invention include C_{5-14} heteroaryl aryl groups, suitably include C_{5-10} heteroaryl

groups.

[0029] The term "C_{2-22}acyl" as used herein refers to a group represented by formula -(C=O)R wherein R represents a C_{1-22} alkyl group as defined here above. Generally, C_{2-22}acyl groups which may be present on the compounds of use in the present invention include C_{2-6}acyl groups, C_{6-22}acyl groups, and C_{12-22}acyl groups. Examples of such C_{2-6}acyl are methyl carbonyl, ethyl carbonyl, and butyl carbonyl. Examples of C_{12-22}acyl groups include palmitinyl carbonyl and stearyl carbonyl.

[0030] The term "C_{1-22}alkoxy" as used herein refers to a group represented by formula -O-R, wherein R represents a C_{1-22} alkyl group as defined here above. Generally, C_{1-22}alkoxy groups which may be present on the compounds of use in the present invention include C_{1-6}alkoxygroups C_{6-22}alkoxy groups and C_{12-22}alkoxy groups. Examples of C_{1-6}alkoxy groups are methoxy and ethoxy. Examples of C_{12-22}alkoxy groups include palmitinyloxy and stearyloxy.

[0031] The term "C_{1-22}heteroalkyl" as used herein refers to a C_{1-22} alkyl as defined above wherein one or more carbon atoms are replaced by one or more oxygen or nitrogen atom. Generally, C_{1-22}heteroalkyl groups which may be present on the compounds of use in the present invention include C_{1-6}heteroalkyl groups, C_{6-22}heteroalkyl groups and C_{12-22}heteroalkyl groups. Examples of C_{1-22}heteroalkyl include oligomers of ethylene glycol.

[0032] The term "hydroxysulphonyl" as used herein refers to a group represented by formula - $S(=O)_2$-OH.

[0033] The term "C_{1-22}alkyl sulphonyl" as used herein refers to a group represented by formula formula -$S(=O)_2$-R, wherein R represents a C_{1-22} alkyl group as defined here above. Generally, C_{1-22} alkyl sulphonyl groups which may be present on the compounds of use in the present invention include C_{1-6} alkyl sulphonyl groups and C_{6-22} alkyl sulphonyl groups, C_{12-22} alkyl sulphonyl groups. Examples of C_{1-22}alkyl sulphonyl include methyl sulphonyl, ethyl sulphonyl, and tert-butyl sulphonyl.

[0034] The term "C_{5-22}aryl sulphonyl" as used herein refers to a group represented by formula - $S(=O)_2$-R', wherein R' represents a C_{5-22} aryl group as defined here above. Examples of C_{5-22}aryl sulphonyl include phenyl sulphonyl and tolyl sulphonyl.

[0035] The term "C_{1-22} alkylamino" as used herein refers to a group represented by formula -NH-R, wherein R represents a C_{1-22} alkyl group as defined here above. Generally, C_{1-22}alkylamino groups which may be present on the compounds of use in the present invention include C_{1-6}alkylamino groups, C_{6-22}alkylamino groups and C_{12-22}alkylamino groups. Examples of C_{1-22}alkyl amino include methylamino, ethylamino, and butylamino.

[0036] The term C_{1-22} alkoxy amino as used herein refers to a group represented by formula -NH-OR wherein R represents a C_{1-22} alkyl group as defined here above. Generally, C_{1-22}alkoxyamino groups which may be present on the compounds of use in the present invention include C_{1-6}alkoxyamino groups, C_{6-22}alkoxyamino groups and C_{12-22}alkoxyamino groups. Examples of C_{1-22}alkyl amino include methylamino, ethylamino, and butylamino.

[0037] The term "di(C_{1-22} alkyl)amino" as used herein refers to by formula -NRR' wherein R and R' represent independently a C_{1-22} alkyl group as defined here above. Generally, di(C_{1-22} alkyl)amino groups which may be present on the compounds of use in the present invention include di(C_{1-6} alkyl)amino, di(C_{6-22} alkyl)amino and di(C_{12-22} alkyl)amino. Examples of di(C_{1-22}alkyl) amino include dimethyl amino, (methyl)(ethyl)amino, diethyl amino, propyl amino and butyl amino.

[0038] The term "C_{1-22}heteroalkylamino" as used herein refers to a group represented by formula - NH-R, wherein R represents a C_{1-22} heteroalkyl group as defined here above. Generally, C_{1-22}heteroalkylamino groups which may be present on the compounds of use in the present invention include C_{1-6}heteroalkylamino, C_{6-22}heteroalkylamino and C_{12-22}heteroalkylamino.

[0039] The term "di(C_{1-22}heteroalkyl)amino" as used herein refers to by formula -NRR' wherein R and R' represent independently a C_{1-22} heteroalkyl group as defined here above. Generally, di(C_{1-22} heteroalkyl)amino groups which may be present on the compounds of use in the present invention include di(C_{1-6} heteroalkyl)amino, di(C_{6-22} heteroalkyl)amino and di(C_{12-22} heteroalkyl)amino.

[0040] The term "C_{1-10} alkylene chain" refers to a divalent straight or branched alkylene chain containing 1 to 10 carbon atoms. Typical examples of "C_{1-10} alkylene chain" include methylene, ethylene, propylene and butylene.

[0041] The term "amino carbonyl" as used herein refers to a group represented by formula -CON(R_aR_b) wherein the carbon of -CO binds to nitrogen of the cysteine/cystine analog and wherein R_a and R_b independently from each other represents a C_{1-22}alkyl as defined above.

[0042] In one embodiment according to the present invention, the cysteine/cystine analogs are selected from cysteine analogs represented by formula 1, wherein R1, R2, R3, R4, R5, R6 R7 and L are as defined here above. In a particular aspect of this embodiment, R7 represents hydrogen.

[0043] In another embodiment according to the present invention, the cysteine/cysteine analogs are selected from cystine analogs represented by formula 2, wherein R1, R2, R3, R4, R5, R6, R7 and L are as defined here above.

[0044] Generally, R1, R2, R4 and R5 independently represent hydrogen, C_{2-22}acyl, C_{1-22}alkyl which group is optionally substituted by one or two substituents selected from hydroxy and C_{1-22}alkoxy; or C_{1-22} heteroalkyl.

[0045] Generally, R3 and R6 independently represent hydroxy; NH_2; C_{1-22}alkoxy wherein one or more carbons of the C_{1-22} alkyl may be optionally replaced by an aryl or an heteroaryl; C_{1-22} alkylamino which group is optionally substituted

by a hydroxy; or di(C_{1-22} alkyl)amino which is optionally substituted by a hydroxy.

[0046] Generally, R7 represents hydrogen.

[0047] Generally, L represents a C_{1-4}alkylene chain, optionally substituted, by one or more C_{1-6} alkyl, preferably, two methyl groups.

[0048] Suitably, R1 represents hydrogen or C_{2-22}acyl. Typically, R1 represents hydrogen or C_{2-6}acyl. Illustratively, R1 represents hydrogen or acetyl. In a particular embodiment R1 represents hydrogen.

[0049] Suitably, R2 represents hydrogen or C_{2-22} acyl. Typically, represents hydrogen or C_{2-6}acyl. Illustratively, R2 represents hydrogen or acetyl.

[0050] Suitably, R3 represents hydroxy or C_{1-22}alkoxy. Typically, R3 hydroxy or C_{1-6}alkoxy. Illustratively, R3 represents hydroxy or methoxy.

[0051] Suitably, R4 represents hydrogen or C_{2-22} acyl. Typically, R4 represents hydrogen or C_{2-6}acyl. Illustratively, R4 represents hydrogen or acetyl. In a particular embodiment, R4 represents hydrogen.

[0052] Suitably, R5 represents hydrogen or C_{2-22} acyl. Typically, R5 represents hydrogen or hydrogen or C_{2-6}acyl. Illustratively, R5 represents acetyl.

[0053] Suitably, R6 represents hydroxy or C_{1-22}alkoxy. Typically, R6 represents hydroxy or C_{1-6}alkoxy. Illustratively, R6 represents hydroxy or methoxy.

[0054] Suitably, L represents methylene or ethylene. Illustratively, L represents methylene.

[0055] In one embodiment, the cysteine/cystine analogs comprises one or more compounds selected from the compounds represented by formula 1, and salts thereof, wherein L, R1, R2, R3 and R7 areas defined above,
with the provisos

(i) that if one of the R1 or R2 groups, represents, C_{6-22}acyl, C_{6-22}alkyl which group is optionally substituted by one or two substituents selected from hydroxy and C_{1-22}alkoxy; C_{6-22} heteroalkyl, hydroxysulphonyl, C_{6-22}alkylsulphonyl, or C_{5-22}aryl sulphonyl; then
the remaining R1 or R2 group represents independently hydrogen, amino carbonyl, C_{2-6}acyl, C_{1-6}alkyl which group is optionally substituted by one or two substituents selected from hydroxy and C_{1-6}alkoxy; C_{1-6} heteroalkyl, hydroxysulphonyl, C_{1-6}alkylsulphonyl, or C_{5-10}aryl sulphonyl; and
R3 represents hydroxy; NH_2; C_{1-6}alkoxy wherein one or more carbons of the C_{1-22} alkyl may be optionally replaced by an aryl or an heteroaryl; C_{1-6} alkylamino which group is optionally substituted by a hydroxy or a C_{1-6} alkyl; hydroxy amino; C_{1-6} alkoxy amino; C_{1-6} alkylamino wherein one or more carbons of the C_{1-6}alkyl are replaced by an aryl or an heteroaryl; C_{1-6}heteroalkylamino; di(C_{1-6} alkyl)amino which is optionally substituted by a hydroxy; di(C_{1-6}alkyl)amino wherein one or more carbons of the C_{1-6}alkyl are replaced by an aryl or heteroaryl; or di(C_{1-6}heteroalkyl)amino; and

(ii) that if
R3 represents C_{6-22}alkoxy wherein one or more carbons of the C_{6-22} alkyl may be optionally replaced by an aryl or an heteroaryl; C_{6-22} alkylamino which group is optionally substituted by a hydroxy or a C_{1-22} alkyl; hydroxy amino; C_{6-22} alkoxy amino; C_{6-22} alkylamino wherein one or more carbons of the C_{1-22}alkyl are replaced by an aryl or an heteroaryl; C_{6-22}heteroalkylamino; di(C_{6-22} alkyl)amino which is optionally substituted by a hydroxy; di(C_{6-22}alkyl)amino wherein one or more carbons of the C_{6-22}alkyl are replaced by an aryl or heteroaryl; or di(C_{6-22}heteroalkyl)amino,
then R1 and R2 independently represents hydrogen, amino carbonyl, C_{2-6}acyl, C_{1-6}alkyl which group is optionally substituted by one or two substituents selected from hydroxy and C_{1-6}alkoxy; C_{1-6} heteroalkyl, hydroxysulphonyl, C_{1-6}alkylsulphonyl, or C_{5-10}aryl sulphonyl.

[0056] In one embodiment, the cysteine/cystine analogs comprise or consist of the compound represented by formula 2, and salts thereof, wherein L, R1, R2, R3, R4, R5, R6 and R7 are as defined above, with the provisos:

(i) that if
one of the of R1 andR2 groups represents C_{6-22}acyl, C_{6-22}alkyl which group is optionally substituted by one or two substituents selected from hydroxy and C_{1-22}alkoxy; C_{6-22} heteroalkyl, hydroxysulphonyl, C_{6-22}alkylsulphonyl, or C_{5-22}aryl sulphonyl; and if one of the R4 and R5 groups represents C_{6-22}acyl, C_{6-22}alkyl which group is optionally substituted by one or two substituents selected from hydroxy and C_{1-22}alkoxy; C_{6-22} heteroalkyl, hydroxysulphonyl, C_{6-22}alkylsulphonyl, or C_{5-22}aryl sulphonyl, then
the remaining R1, R2, R4, or R5 groups independently represent hydrogen, amino carbonyl, C_{2-6}acyl, C_{1-6}alkyl which group is optionally substituted by one or two substituents selected from hydroxy and C_{1-6}alkoxy; C_{1-6} heteroalkyl, hydroxysulphonyl, C_{1-6}alkylsulphonyl, or C_{5-10}aryl sulphonyl; and
R3 and R6 independently represents hydroxy; NH_2; C_{1-6}alkoxy wherein one or more carbons of the C_{1-22} alkyl may

be optionally replaced by an aryl or an heteroaryl; C_{1-6} alkylamino which group is optionally substituted by a hydroxy or a C_{1-6} alkyl; hydroxy amino; C_{1-6} alkoxy amino; C_{1-6} alkylamino wherein one or more carbons of the C_{1-6}alkyl are replaced by an aryl or an heteroaryl; C_{1-6}heteroalkylamino; di(C_{1-6} alkyl)amino which is optionally substituted by a hydroxy; di(C_{1-6}alkyl)amino wherein one or more carbons of the C_{1-6}alkyl are replaced by an aryl or heteroaryl; or di(C_{1-6}heteroalkyl)amino.;

(ii) that if only if one of R1, R2, R4 and R5 groups independently represent C_{6-22}acyl, C_{6-22}alkyl which group is optionally substituted by one or two substituents selected from hydroxy and C_{1-22}alkoxy; C_{6-22} heteroalkyl, hydroxysulphonyl, C_{6-22}alkylsulphonyl, or C_{5-22}aryl sulphonyl; then
one of R3 and R6 may r represent C_{6-22}alkoxy wherein one or more carbons of the C_{1-22} alkyl may be optionally replaced by an aryl or an heteroaryl; C_{6-22} alkylamino which group is optionally substituted by a hydroxy or a C_{1-22} alkyl; C_{6-22} alkoxy amino; C_{6-22} alkylamino wherein one or more carbons of the C_{1-22}alkyl are replaced by an aryl or an heteroaryl; C_{6-22}heteroalkylamino; di(C_{6-22} alkyl)amino which is optionally substituted by a hydroxy; di(C_{6-22}alkyl)amino wherein one or more carbons of the C_{6-22}alkyl are replaced by an aryl or heteroaryl; or di(C_{6-22}heteroalkyl)amino;

(iii) that if none of the R1, R2, R4 and R5 groups represents C_{6-22}acyl, C_{6-22}alkyl which group is optionally substituted by one or two substituents selected from hydroxy and C_{1-22}alkoxy; C_{6-22} heteroalkyl, hydroxysulphonyl, C_{6-22}alkylsulphonyl, or C_{5-22}aryl sulphonyl; then
R3 and R6 may both represent independently C_{6-22}alkoxy wherein one or more carbons of the C_{1-22} alkyl may be optionally replaced by an aryl or an heteroaryl; C_{6-22} alkylamino which group is optionally substituted by a hydroxy or a C_{1-22} alkyl; C_{6-22} alkoxy amino; C_{6-22} alkylamino wherein one or more carbons of the C_{1-22}alkyl are replaced by an aryl or an heteroaryl; C_{6-22}heteroalkylamino; di(C_{6-22} alkyl)amino which is optionally substituted by a hydroxy; di(C_{6-22}alkyl)amino wherein one or more carbons of the C_{6-22}alkyl are replaced by an aryl or heteroaryl; or di(C_{6-22}heteroalkyl)amino.

[0057] The present invention also includes within its scope salts of cysteine/cystine analogs of formula 1 and 2.

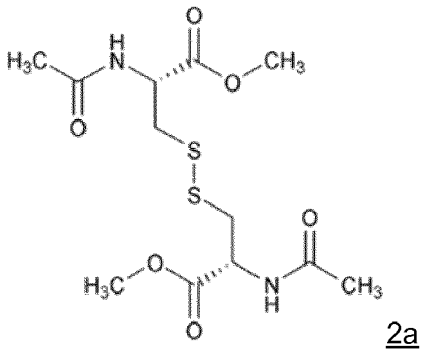
[0058] Salts according to the invention may be formed by reaction of a hydroxy group present on the cysteine/cystine analogs. Such salts include alkali metal salts, for example sodium, potassium or lithium salts; alkali earth metal salts, for example magnesium or calcium salts; ammonium salts, for example tetra alkyl or aryl ammonium salts; sulphonium salts, for example trialkyl or aryl sulphonium; and phosphonium salts, for example tetra alkyl or aryl phosphonium salts.

[0059] Alternatively, such salts may be formed by reaction of an amino group present on the cysteine/cysteine analogs. Such salts typically result from the reaction of the amino group with an inorganic acid or an organic acid and include mono- or di- HCl salts, H_2SO_4 salts, H_3PO_4 salts, acetate and fumarate.

[0060] Suitably, the cysteine/cystine analogs as defined here above have the same chirality as L-cysteine.

[0061] In a particular embodiment of the present invention the cysteine/cysteine analogs are selected from N,N'-diacetyl-L-cystine-dimethylester, N-Acetyl-L-cysteine and N,N'-Diacetyl-L-cystine.

[0062] In a particular aspect of this embodiment, the cysteine/cystine analogs consists of, N,N'-diacetyl-L-cystine-dimethylester represented by formula 2a ((Ac-Cys-OMe)$_2$.



2a

[0063] Cysteine/cysteine analogs according to the present invention may be commercially available or may be synthesized from L-cysteine or L-cystine by methods known to the skilled in the art.

Cell culture

[0064] As it will be apparent from the description of the invention hereinafter, in most embodiments of the method of the invention, the cell culture medium is supplemented with cysteine and/or cystine, i.e. such supplementation may be performed with:

- cysteine; or
- cystine; or
- cysteine and cystine

[0065] Cysteine and cystine in the cell culture medium are in constant equilibrium wherein two molecules of cysteine oxidize into a molecule of cystine and reduce back to two molecules of cysteine.

[0066] The term "cell culture" or grammatical variations thereof includes, but it is not limited to, a plurality of host cells, preferably mammalian host cells, suitably engineered and/or manipulated to express (i.e. to produce) one or more recombinant polypeptides maintained or grown in cell culture medium for a particular period of time, e.g. the production phase.

[0067] The term "production phase" according to the present invention comprises that stage of cell culturing during the process for manufacturing a recombinant protein when the cells express (i.e. produce) the recombinant polypeptide(s). The production phase begins when the titer of the desired product increases and ends with harvest of the cells or the cell culture fluid or supernatant. Typically, at the beginning of the production phase, the cell culture is transferred to a production vessel, such as a bioreactor. Harvest is the step during which the cell culture fluid is removed from the e.g. production vessel, in order for the recombinant protein e.g. the recombinant antibody, to be recovered and purified in subsequent steps.

[0068] Preferred host cells are mammalian host cells, most preferably Chinese Hamster Ovary (CHO) cells. Mammalian cells, and in particular CHO cells, may be cultured in any medium that will support their growth and expression of the recombinant polypeptide, preferably the medium is a medium that is free of animal-derived products such as animal serum and peptone. There are different cell culture media available to the person skilled in the art, each medium comprising different combinations of vitamins, amino acids, hormones, growth factors, ions, buffers, nucleosides, glucose or an equivalent energy source, present at appropriate concentrations to enable cell growth and protein production. Suitable media have e.g. been described in WO98/08934 and US2006/0148074 (both incorporated herein in their entirety). Further suitable commercially available media that could be used in the present invention or be modified to fulfil the cysteine/cysteine analog and/or cysteine and/or cystine requirements include, but are not limited to, AmpliCHO CD medium, Dynamis™ Medium, EX-CELL® Advanced™ CHO Fed-batch System, CD FortiCHO™ medium, CP OptiCHO™ medium, Minimum Essential Media (MEM), BalanCD® CHO Growth A Medium, ActiPro™ medium, DMEM-Dulbecco's Modified Eagle Medium and RPMI-1640 medium.

[0069] In a preferred embodiment of the method of the invention, wherein said cell culture medium comprises:

(a) cysteine/cystine analogs; and

(b) cysteine and/or cystine,

wherein the molar ratio of (a) to (b) is between 1:18 and 18:1.

[0070] Preferably, said molar ratio of (a) to (b) is between 1:15 and 15:1, e.g. between 1:12 and 12:1, such as between 1:10 and 10:1, e.g. between 1:8 and 8:1, such as between 1:6 and 6:1, e.g. between 1:4 and 4:1, such as between 1:3 and 3:1, e.g. between 1:2 and 2:1, such as between 1.5:1 and 1:1.5, e.g. a molar ratio of 1:1.

[0071] In another preferred embodiment, the method comprises the steps of:

(i) inoculating said host cells in a basal medium, wherein the basal medium optionally comprises an initial amount of:

(a) cysteine/cystine analogs; and/or

(b) cysteine and/or cystine,

(ii) progressing the culture through a production phase wherein the recombinant protein is produced by the cells, wherein, during said production phase, the cell culture medium is supplemented with:

(a) cysteine/cystine analogs; and/or

(b) cysteine and/or cystine,

wherein (a) and (b) may be added simultaneously or sequentially,
wherein, when the contents of the basal medium and the total supplements added are added up, the molar ratio of (a) to (b) is between 1:18 and 18:1.

[0072] Preferably, in the above-mentioned embodiments, said molar ratio of (a) to (b) is between 1:15 and 15:1, e.g. between 1:12 and 12:1, such as between 1:10 and 10:1, e.g. between 1:8 and 8:1, such as between 1:6 and 6:1, e.g. between 1:4 and 4:1, such as between 1:3 and 3:1, e.g. between 1:2 and 2:1, such as between 1.5:1 and 1:1.5, e.g. a molar ratio of 1:1.

[0073] In one embodiment, the concentration of (b) in said basal medium is equivalent to between 0.05 and 5 mmol/L of cysteine, such as between 0.1 and 1 mmol/L, e.g. between 0.2 and 0.6 mmol/L.

[0074] "equivalent to X mol of cysteine" herein indicates that if dimer forms, such as cystine or cystine analogs are used, they should be counted double for the purposes of calculating the amount to be used or added. E.g. 1 mmol/L of cystine is equivalent to 2 mmol/L of cysteine.

[0075] In another embodiment, during the production phase, the medium is supplemented with (a) cysteine/cystine analogs and (b) cysteine and/or cystine, wherein the sum of (a) and (b) added to the culture over the entire production phase is equivalent to between 1 and 75 mmol/L of cysteine, such as between 1 and 50 mmol/L, e.g. between 1 and 20 mmol/L, such as between 2 and 20 mmol/L, e.g. between 4 and 10 mmol/L.

[0076] In another embodiment, during the production phase, the medium is supplemented daily with (a) cysteine/cystine analogs and (b) cysteine and/or cystine, wherein the daily addition brings the concentration of (a) + (b) to a concentration equivalent to between 0.05 and 5 mmol/L of cysteine, such as between 0.1 and 1 mmol/L.

[0077] The production phase is operated preferably in a fed-batch mode, but any other mode such as batch, perfusion or chemostat modes can be used as an alternative.

[0078] Cell culture can take place in any suitable container such as a shake flask or a bioreactor, which may or may not be operated in a fed-batch mode depending on the scale of production required. These bioreactors may be either stirred-tank or air-lift reactors. Preferable, the production phase is carried out in a bioreactor, preferably with a volume of equal or more than 50 L, equal or more than 100 L, equal or more than 500 L, equal or more than 1000 L, equal or more than 2,000 L, equal or more than 5,000 L, equal or more than 10,000 L or equal or more than 20,000 L.

[0079] Preferably, the recombinant protein is produced during a production phase, wherein the production phase preferably has a duration of at least 7 days, more preferably at least 14 days.

[0080] In preferred embodiments, the culture is supplemented daily in the production phase.
In one embodiment of the method of the invention, the cell culture medium is supplemented with:

- cysteine or cystine up to a total amount of from 10 wt% to 30 wt% of the expected total amount of recombinant protein produced; and/or
- tryptophan up to a total amount of from 8 wt% to 35 wt% of the expected total amount of recombinant protein produced.

[0081] The total amount of cysteine or cystine and/or tryptophan added may be expressed herein as a percentage of the total amount of recombinant polypeptide produced. The term "wt%" as used herein refers to percentage of weight. "Total" refers to the total amount as determined at the end of the production phase, i.e. the total amount of cysteine or cystine and/or tryptophan added over the course of the production phase and the total amount of recombinant protein produced over the course of the production phase, wherein the total amount of recombinant protein produced is measured at the end of the production phase.

[0082] The total amount of cysteine or cystine or tryptophan added is calculated as a function of the feed rate (or feed volume) and the concentration of cysteine or cystine or tryptophan in that feed and the concentration of cysteine or cystine or tryptophan in the medium where the feed is added per volume of feed added. The quantity of recombinant polypeptide produced is calculated as a function of the final volume of the cell culture medium and the final recombinant polypeptide titer. The ratio of these two calculated parameters is the total amount of cysteine or cystine and/or tryptophan added per quantity of recombinant polypeptide produced.

[0083] The host cells may initially (in step a.) be grown in a cell culture medium which may or may not already include cysteine/cystine analogs, cysteine, cystine and/or tryptophan. If the cell culture medium already includes an initial amount of cysteine/cystine analogs, cysteine, cystine and/or tryptophan, then the total amount will include this initial amount.

[0084] In one embodiment of the process of the invention, the cell culture medium is supplemented with cysteine or cystine up to a total amount of from 12.06 wt% to 28.03 wt% of the expected total amount of recombinant polypeptide produced, such as a total amount of from 12 wt% to 28 wt%, e.g. from 12 wt% to 25 wt%, such as from 12 wt% to 20 wt% of the expected total amount of recombinant polypeptide produced.

[0085] In another embodiment of the process of the invention, wherein the cell culture medium is supplemented with tryptophan up to a total amount of from 8.84 wt% to 32.06 wt% of the expected total amount of recombinant polypeptide

produced, such as a total amount of from 8 wt% to 30 wt%, e.g. from 8 wt% to 25 wt%, such as from 8 wt% to 20 wt% of the expected total amount of recombinant polypeptide produced.
In another embodiment of the method of the invention,

- the cysteine or cystine concentration in the cell culture does not exceed 0.9 g/L at any time point during the production phase, preferably wherein the cysteine or cystine concentration in the cell culture does not exceed 0.3 g/L at any time point during the production phase, and/or
- the tryptophan concentration in the cell culture does not exceed 0.6 g/L at any time point during the production phase, preferably wherein the tryptophan concentration in the cell culture does not exceed 0.3 g/L at any time point during the production phase.

[0086] In a further embodiment of the method of the invention,

- the total amount of cysteine or cystine provided during the process is from 2.9 to 12 g/(10^{12} cells), such as from 2.9 to 7 g/(10^{12} cells), e.g. from 5.6 to 7 g/(10^{12} cells), wherein cells refers to the expected integral viable cell count at the end of the production phase, and/or
- the total amount of tryptophan provided during the process is from 2.5 to 7 g/(10^{12} cells), such as from 2.5 to 3.5 g/(10^{12} cells/L), wherein cells refers to the expected integral viable cell count at the end of the production phase.

[0087] It should be understood that the skilled person would know how to measure the amount of cysteine or cystine and/or tryptophan added to and/or present in a cell culture at a specific phase, such as the production phase. Similarly, the skilled person would know how to measure the total amount of recombinant polypeptide produced by a cell culture and consequently apply the teaching of the present invention to achieve the desired technical effect.
[0088] In order to design a process wherein the amounts of cysteine or cystine and/or tryptophan per total amount of recombinant polypeptide produced are kept within certain ranges, it may be required to perform one or more initial experiments to determine the approximate levels of recombinant polypeptide produced by particular host cells under particular culturing conditions. Once the approximate total levels of recombinant polypeptide produced are known, a process according to the invention can be designed wherein the amounts of cysteine or cystine and/or tryptophan per total amount of recombinant polypeptide produced are kept within the specified ranges,
[0089] Various strategies may be employed for reaching the total amount of cysteine/cystine analogs, cysteine, cystine and/or tryptophan in the cell culture medium during the production phase. In one embodiment, the total amount may be reached by adding cysteine/cystine analogs, cysteine, cystine and/or tryptophan right at the beginning of the production phase, for example only once or as being already included in the production cell culture medium. In another embodiment, the total amount may be reached by the summation of additions, for example daily addition or continuous addition, during the production phase. In yet another embodiment, the total amount may be reached by a combination of the initial cysteine/cystine analogs, cysteine, cystine and/or tryptophan concentration in the cell culture fluid at the start of the production phase, and by way of additions.
[0090] Accordingly, in one embodiment of the process of the invention, the total amount of cysteine/cystine analogs, cysteine, cystine and/or tryptophan in the cell culture medium is reached by adding cysteine/cystine analogs, cysteine, cystine and/or tryptophan to the cell culture medium:

a. at the beginning of the production phase,
b. once or multiple times at any time point during the production phase,
c. through continuous addition during the production phase, or
d. in any combination of a., b. and c.

[0091] In a further independent aspect, the invention relates to a cell culture medium suitable for culturing mammalian cells comprising N,N'-diacetyl-L-cystine-dimethylester.

Recombinant polypeptides

[0092] The process of the invention can be used to produce any type of recombinant protein or polypeptide, including for example, peptides or larger polypeptides having significant tertiary structure as well as e.g. glycoproteins and multimeric proteins.
[0093] In some embodiments, the recombinant protein produced is a protein which, when produced under standard conditions, would result in a colored preparation at high concentration. Such coloration can be reduced or avoided using the method of the invention. Thus, in a preferred embodiment of the method of the invention, the recombinant protein is a protein which is not colorless at a concentration of 10 mg/ml or more, such as 50 mg/ml or more, when produced

by host cells grown in a cell culture medium not comprising cysteine/cystine analogs, wherein color e.g. is determined as described in the Examples herein. Protein preparations, such as antibodies, that are to be administered subcutaneously to a patient, often have even higher proteins concentrations of e.g. 100 mg/ml or more, or even more than 150 mg/ml. At such concentrations, undesirable coloration frequently becomes a problem. Thus, in another preferred embodiment, the recombinant protein is a protein which, when produced by host cells grown in a cell culture medium not comprising cysteine/cystine analogs is not colorless at a concentration of 100 mg/ml or more, such as 150 mg/ml or more.

[0094] In a preferred embodiment, the recombinant protein produced in the process according to the invention is an antibody or an antigen-binding fragment thereof.

[0095] The term "antibody" or "antibodies" as used herein includes e.g. both monoclonal and polyclonal antibodies as well as both monospecific and multispecific, such as bispecific, antibodies. Antibody" or "antibodies" include antibodies' of any species, in particular of mammalian species, typically having two heavy chains and two light chains, human antibodies of any isotype, including IgA_1, IgA_2, IgD, IgG_1, IgG_{2a}, IgG_{2b}, IgG_3, IgG_4 IgE, and IgM and modified variants thereof, non-human primate antibodies, e.g. from chimpanzee, baboon, rhesus or cynomolgus monkey, rodent antibodies, e.g. from mouse, rat or rabbit; goat or horse antibodies, and derivatives thereof, or of bird species such as chicken antibodies or of fish species such as shark antibodies. The term "antibody" or "antibodies" also refers to "chimeric" antibodies in which a first portion of at least one heavy and/or light chain antibody sequence is from a first species and a second portion of the heavy and/or light chain antibody sequence is from a second species. Chimeric antibodies of interest herein include "primatized" antibodies comprising variable domain antigen-binding sequences derived from a non-human primate (e.g. Old-World Monkey, such as baboon, rhesus or cynomolgus monkey) and human constant region sequences. "Humanized" antibodies are chimeric antibodies that contain a sequence derived from non-human antibodies. For the most part, humanized antibodies are human antibodies (recipient antibody) in which residues from a hypervariable region of the recipient are replaced by residues from a hypervariable region or complementarity determining region (CDR) of a non-human species (donor antibody) such as mouse, rat, rabbit, chicken or non-human primate, having the desired specificity, affinity, and activity. In most instances residues of the human (recipient) antibody outside of the CDR; i.e. in the framework region (FR), are additionally replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues that are not found in the recipient antibody or in the donor antibody. These modifications are made to further refine antibody performance. Humanization reduces the immunogenicity of non-human antibodies in humans, thus facilitating the application of antibodies to the treatment of human diseases. Humanized antibodies and several different technologies to generate them are well known in the art. The term "antibody" or "antibodies" also refers to human antibodies, which can be generated as an alternative to humanization. For example, it is possible to produce transgenic animals (e.g., mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of production of endogenous murine antibodies. For example, it has been described that the homozygous deletion of the antibody heavy-chain joining region (JH) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies with specificity against a particular antigen upon immunization of the transgenic animal carrying the human germ-line immunoglobulin genes with said antigen. Technologies for producing such transgenic animals and technologies for isolating and producing the human antibodies from such transgenic animals are known in the art. Alternatively, in the transgenic animal; e.g. mouse, only the immunoglobulin genes coding for the variable regions of the mouse antibody are replaced with corresponding human variable immunoglobulin gene sequences. The mouse germline immunoglobulin genes coding for the antibody constant regions remain unchanged. In this way, the antibody effector functions in the immune system of the transgenic mouse and consequently the B cell development are essentially unchanged, which may lead to an improved antibody response upon antigenic challenge in vivo. Once the genes coding for a particular antibody of interest have been isolated from such transgenic animals the genes coding for the constant regions can be replaced with human constant region genes in order to obtain a fully human antibody. The term "antibody" or "antibodies" as used herein, also refers to an aglycosylated antibody.

[0096] The term "antigen-binding fragment thereof" or grammatical variations thereof as used herein refers to an antibody fragment. A fragment of an antibody comprises at least one heavy or light chain immunoglobulin domain as known in the art and binds to one or more antigen(s). Examples of antibody fragments according to the invention include Fab, Fab', $F(ab')_2$, and Fv and scFv fragments; as well as diabodies, triabodies, tetrabodies, minibodies, domain antibodies(dAbs), such as sdAbs, VHH or camelid antibodies (e.g. from camels or llamas such as Nanobodies™) and VNAR fragments, single-chain antibodies, bispecific, trispecific, tetraspecific or multispecific antibodies formed from antibody fragments or antibodies, including but not limited to Fab-Fv or Fab-Fv-Fv constructs. When used herein, antibody fragments also include molecules which comprises non-immunoglobulin-derived sequences in addition to immunoglobulin domains, e.g. in the form of fusion proteins. Antibody fragments as defined above are known in the art.

[0097] In a particularly preferred embodiment, the antibody or antigen-binding fragment thereof produced through the methods according to the invention is (Table 1):

1) an antibody or antigen-binding fragment thereof which

a. comprises CDR-H1 having the sequence as defined in SEQ ID NO:1; CDR-H2 having the sequence as defined in SEQ ID NO:2; CDR-H3 having the sequence as defined in SEQ ID NO:3; CDR-L1 having the sequence as defined in SEQ ID NO:4; CDR-L2 having the sequence as defined in SEQ ID NO:5 and CDR-L3 having the sequence as defined in SEQ ID NO:6; or
b. comprises a light variable region having the sequence as defined in SEQ ID NO: 7 and a heavy variable region having the sequence as defined in SEQ ID NO: 8; or
c. comprises a light variable region having at least 80% identity or similarity, preferably 90% identity or similarity to the sequence as defined in SEQ ID NO: 7 and a heavy variable region having at least 80% identity or similarity, preferably 90% identity or similarity to the sequence as defined in SEQ ID NO: 8;
d. comprises a light variable region having the sequence as defined in SEQ ID NO: 7 and a heavy chain having the sequence as defined in SEQ ID NO: 11; or
e. comprises a light variable region having at least 80% identity or similarity, preferably 90% identity or similarity to the sequence as defined in SEQ ID NO: 7 and a heavy chain having at least 80% identity or similarity, preferably 90% identity or similarity to the sequence as defined in SEQ ID NO: 11; or

2) an antibody which comprises a light chain having the sequence as defined in SEQ ID NO: 9 and a heavy chain having the sequence as defined in SEQ ID NO: 10; or
3) an antibody which comprises a light chain having at least 80% identity or similarity, preferably 90% identity or similarity to the sequence as defined in SEQ ID NO: 9 and a heavy chain having at least 80% identity or similarity, preferably 90% identity or similarity to the sequence as defined in SEQ ID NO: 10.

[0098] Complementarity determining regions ("CDR") are defined herein according to the Kabat definition. The Kabat definition is a standard for numbering the residues in an antibody and it is typically used to identify CDR regions (Kabat et al., (1991), 5th edition, NIH publication No. 91-3242).

CDR-H1 SEQ ID NO: 1	GFTFSNYGMV
CDR-H2 SEQ ID NO: 2	YIDSDGDNTYYRDSVKG
CDR-H3 SEQ ID NO: 3	GIVRPFLY
CDR-L1 SEQ ID NO: 4	KSSQSLVGASGKTYLY
CDR-L2 SEQ ID NO: 5	LVSTLDS
CDR-L3 SEQ ID NO: 6	LQGTHFPHT
Light variable region SEQ ID NO:7	DIQMTQSPSS LSASVGDRVT ITCKSSQSLV GASGKTYLYW LFQKPGKAPK RLIYLVSTLD SGIPSRFSGS GSGTEFTLTI SSLQPEDFAT YYCLQGTHFP HTFGQGTKLE IK
Heavy variable region SEQ ID NO:8	EVPLVESGGG LVQPGGSLRL SCAVSGFTFS NYGMVWVRQA PGKGLEWVA IDSDGDNTYY RDSVKGRFTI SRDNAKSSLY LQMNSLRAED TAVYYCTTG VRPFLYWGQG TLVTVS

(continued)

Light chain SEQ ID NO: 9	DIQMTQSPSS LSASVGDRVT ITCKSSQSLV GASGKTYLYW LFQKPGKAPK RLIYLVSTLD SGIPSRFSGS GSGTEFTLTI SSLQPEDFAT YYCLQGTHFP HTFGQGTKLE IKRTVAAPSV FIFPPSDEQL KSGTASVVCL LNNFYPREAK VQWKVDNALQ SGNSQESVTE QDSKDSTYSL SSTLTLSKAD YEKHKVYACE VTHQGLSSPV TKSFNRGEC
Heavy chain SEQ ID NO: 10	EVPLVESGGG LVQPGGSLRL SCAVSGFTFS NYGMVWVRQA PGKGLEWVAY IDSDGDNTYY RDSVKGRFTI SRDNAKSSLY LQMNSLRAED TAVYYCTTGI VRPFLYWGQG TLVTVSSAST KGPSVFPLAP CSRSTSESTA ALGCLVKDYF PEPVTVSWNS GALTSGVHTF PAVLQSSGLY SLSSVVTVPS SSLGTKTYTC NVDHKPSNTK VDKRVESKYG PPCPPCPAPE FLGGPSVFLF PPKPKDTLMI SRTPEVTCVV VDVSQEDPEV QFNWYVDGVE VHNAKTKPRE EQFNSTYRVV SVLTVLHQDW LNGKEYKCKV SNKGLPSSIE KTISKAKGQP REPQVYTLPP SQEEMTKNQV SLTCLVKGFY PSDIAVEWES NGQPENNYKT TPPVLDSDGS FFLYSRLTVD KSRWQEGNVF SCSVMHEALH NHYTQKSLSL SLGK
Fab heavy chain SEQ ID NO: 11	EVPLVESGGG LVQPGGSLRL SCAVSGFTFS NYGMVWVRQA PGKGLEWVA IDSDGDNTYY RDSVKGRFTI SRDNAKSSLY LQMNSLRAED TAVYYCTTG VRPFLYWGQG TLVTVSSAST KGPSVFPLAP SSKSTSGGTA ALGCLVKDY PEPVTVSWNS GALTSGVHTF PAVLQSSGLY SLSSVVTVPS SSLGTQTYI NVNHKPSNTK VDKKVEPKSC

The recombinant protein or the preferred antibody or antigen-binding fragment thereof may be typically produced by host cells containing a vector encoding the polypeptide or antibody nucleotide sequence. Antibodies or antigen-binding fragment thereof may comprise only a heavy or light chain polypeptide, in which case only a heavy chain or light chain polypeptide coding sequence needs to be used to transfect the cells. For production of products comprising both heavy and light chains, the cells may be transfected with two vectors, a first vector encoding a light chain polypeptide and a second vector encoding a heavy chain polypeptide. Alternatively, a single vector may be used, the vector including sequences encoding light chain and heavy chain polypeptides.

Optional further steps

[0099] The method of the invention optionally further comprises a step of recovering the recombinant protein from the cell culture medium. Subsequently, the recombinant protein may be purified, e.g. if the protein is an antibody, using Protein A chromatography. The method further optionally comprises a step of formulating the purified recombinant protein, e.g. into a formulation with a high protein concentration, such as a concentration of 10 mg/ml or more, e.g. 50 mg/ml or more, such as 100 mg/ml or more, e.g. 150 mg/ml or more. In a further embodiment, the protein is lyophilised or spray-dried. The protein may be administered in a dry form to a patient or be reconstituted into a liquid formation prior to administration.

Products obtained or obtainable by the method of the invention

[0100] In a further aspect, the invention relates to a recombinant protein preparation, such as a bulk recombinant protein preparation, obtainable or obtained by the methods according to the invention. The recombinant proteins, preferably the antibodies or antigen-binding fragments thereof in said preparation so obtained exhibit reduced heterogeneity

as compared to the same recombinant proteins obtained with the same process, but wherein the medium does not include cysteine/cystine analogs. In preferred embodiments, the preparation is colorless.

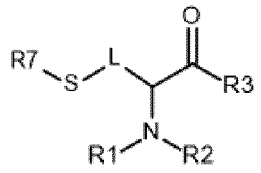
Further aspects and embodiments of the invention:

[0101]

1. A method for reducing the heterogeneity of a population of recombinant proteins produced in cell culture, said method comprising growing host cells producing a recombinant protein in a cell culture medium wherein the cell culture medium comprises one or more cysteine/cystine analogs wherein the reduction of the heterogeneity is obtained without substantially reducing the titer of the recombinant proteins at the end of the production.
2. The method according to embodiment 1, wherein said reduction of heterogeneity comprises reducing

a. color or intensity of color;
b. charge heterogeneity, preferably by reducing acidic peak group species (APG) and/or basic peak group species (BPG), whereby the main charge species substantially does not decrease; and/or
c. amino acid oxidation, preferably methionine oxidation.

3. In one embodiment according to the present invention, the cysteine/cystine analogs comprise or consist of one or more compounds selected from the compounds represented by formula 1 and 2, and salts thereof:



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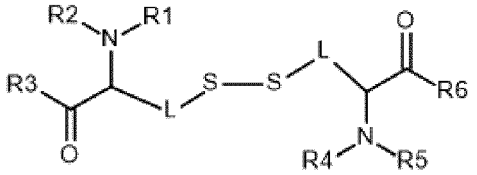

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wherein,

R1, R2, R4 and R5 independently represent hydrogen, amino carbonyl, C_{2-22}acyl, C_{1-22}alkyl which group is optionally substituted by one or two substituents selected from hydroxy and C_{1-22}alkoxy; C_{1-22} heteroalkyl, hydroxysulphonyl, C_{1-22}alkylsulphonyl, or C_{5-22}aryl sulphonyl;
R3 and R6 independently represent hydroxy; NH_2; C_{1-22}alkoxy wherein one or more carbons of the C_{1-22} alkyl may be optionally replaced by an aryl or an heteroaryl; C_{1-22} alkylamino which group is optionally substituted by a hydroxy or a C_{1-22} alkyl; hydroxy amino; C_{1-22} alkoxy amino; C_{1-22} alkylamino wherein one or more carbons of the C_{1-22}alkyl are replaced by an aryl or an heteroaryl; C_{1-22}heteroalkylamino; di(C_{1-22} alkyl)amino which is optionally substituted by a hydroxy; di(C_{1-22}alkyl)amino wherein one or more carbons of the C_{1-22}alkyl are replaced by an aryl or heteroaryl; or di(C_{1-22}heteroalkyl)amino;
R7 represents hydrogen, phosphate or sulphate;
L represents an optionally substituted C_{1-10}alkylene chain; and

with the proviso that the compound is not cysteine or cystine.
4. The method according to embodiment 3, wherein C_{1-22}alkyl refers to aliphatic hydrocarbon groups which may be straight or branched and may comprise 1 to 22 carbon atoms in the chain, such as C_{6-22} alkyl groups, C_{12-22} alkyl groups, C_{1-16} alkyl groups, C_{1-10} alkyl groups and C_{1-6} alkyl groups.
5. The method according to embodiment 3 or 4, wherein C_{5-22}aryl refers to an unsaturated aromatic carbocyclic group of from 5 to 22 carbon atoms having a single ring or multiple condensed rings, such as C_{5-14} aryl groups, C_{5-10} aryl groups.
6. The method according to any of the embodiments 3 to 5, wherein "C_{5-22}heteroaryl" represents aromatic carbocyclic groups of from 5 to 22 carbon atoms having a single ring or multiple condensed rings, wherein one or more of the said carbon atoms have been replaced by one or more heteroatoms selected from oxygen, sulphur and nitrogen and wherein C_{5-22}heteroaryl include C_{5-14} heteroaryl aryl groups, suitably include C_{5-10} heteroaryl groups.

7. The method according to any of the embodiments 3 to 5, wherein C_{2-22}acyl refers to a group represented by formula -(C=O)R wherein R represents a C_{1-22} alkyl group as defined here above, and include C_{2-6}acyl groups, C_{6-22}acyl groups, and C_{12-22}acyl groups.

8. The method according to any of the embodiments 3 to 7, wherein C_{1-22}alkoxy refers to a group represented by formula -O-R, wherein R represents a C_{1-22} alkyl group as defined here above and include C_{1-6}alkoxygroups C_{6-22}alkoxy groups and C_{12-22}alkoxy groups.

9. The method according to any of the embodiments 3 to 8, wherein C_{1-22}heteroalkyl refers to a C_{1-22} alkyl as defined above wherein one or more carbon atoms are replaced by one or more oxygen or nitrogen atom and include C_{1-6}heteroalkyl groups, C_{6-22}heteroalkyl groups and C_{12-22}heteroalkyl groups.

10. The method according to any of the embodiments 3 to 9, wherein hydroxysulphonyl refers to a group represented by formula -$S(=O)_2$-OH.

11. The method according to any of the embodiments 3 to 10, wherein $_{1-22}$alkyl sulphonyl refers to a group represented by formula formula -$S(=O)_2$-R, wherein R represents a C_{1-22} alkyl group as defined here above and include C_{1-6} alkyl sulphonyl groups and C_{6-22} alkyl sulphonyl groups, C_{12-22} alkyl sulphonyl groups.

12. The method according to any of the embodiments 3 to 11, wherein C_{5-22}aryl sulphonyl as used herein refers to a group represented by formula -$S(=O)_2$-R', wherein R' represents a C5-22 aryl group as defined here above.

13. The method according to any of the embodiments 3 to 12, wherein C_{1-22} alkylamino refers to a group represented by formula -NH-R wherein R represents a C_{1-22} alkyl group as defined here above and include C_{1-6}alkylamino groups, C_{6-22}alkylamino groups and C_{12-22}alkylamino groups.

14. The method according to any of the embodiments 3 to 13, wherein C_{1-22} alkoxy amino refers to a group represented by formula -NH-OR wherein R represents a C_{1-22} alkyl group as defined here above and include C_{1-6}alkoxyamino groups, C_{6-22}alkoxyamino groups and C_{12-22}alkoxyamino groups.

15. The method according to any of the embodiments 3 to 14, wherein di(C_{1-22} alkyl)amino refers to by formula -NRR' wherein R and R' represent independently a C_{1-22} alkyl group as defined here above and include di(C_{1-6} alkyl)amino, di(C_{6-22} alkyl)amino and di(C_{12-22} alkyl)amino.

16. The method according to any of the embodiments 3 to 15, wherein C_{1-22}heteroalkylamino refers to a group represented by formula -NH-R wherein R represents a C_{1-22} heteroalkyl group as defined here above and include C_{1-6}heteroalkylamino, C_{6-22}heteroalkylamino and C_{12-22}heteroalkylamino.

17. The method according to any of the embodiments 3 to 16, wherein di(C_{1-22}heteroalkyl)amino refers to by formula -NRR' wherein R and R' represent independently a C_{1-22} heteroalkyl group as defined here above and include di(C_{1-6} heteroalkyl)amino, di(C_{6-22} heteroalkyl)amino and di(C_{12-22} heteroalkyl)amino.

18. The method according to any of the embodiments 3 to 17, wherein C_{1-10} alkylene chain refers to a divalent straight or branched alkylene chain containing 1 to 10 carbon atoms and include methylene, ethylene, propylene and butylene.

19. The method according to any of the embodiments 3 to 18, wherein "amino carbonyl" refers to a group represented by formula -CO-N(R_aR_b) wherein the carbon of-CO binds to nitrogen of the cysteine/cystine analog and wherein R_a and R_b independently from each other represent a C_{1-22}alkyl as defined above.

20. The method according to any of the embodiments 3 to 19, wherein the cysteine/cystine analogs are selected from cysteine analogs represented by formula 1, wherein R1, R2, R3, R4, R5, R6 R7 and L are as defined here above.

21. The method according to any of the embodiments 3 to 20, wherein R7 represents hydrogen.

22. The method according to any of the embodiments 3 to 21, wherein the cysteine/cysteine analogs are selected from cystine analogs represented by formula 2, wherein R1, R2, R3, R4, R5, R6, R7 and L are as defined here above.

23. The method according to any of the embodiments 3 to 22, wherein R1, R2, R4 and R5 independently represent hydrogen, C_{2-22}acyl, C_{1-22}alkyl which group is optionally substituted by one or two substituents selected from hydroxy and C_{1-22}alkoxy; or C_{1-22} heteroalkyl.

24. The method according to any of the embodiments 3 to 23, wherein R3 and R6 independently represent hydroxy; NH_2; C_{1-22}alkoxy wherein one or more carbons of the C_{1-22} alkyl may be optionally replaced by an aryl or an heteroaryl; C_{1-22} alkylamino which group is optionally substituted by a hydroxy; or di(C_{1-22} alkyl)amino which is optionally substituted by a hydroxy.

25. The method according to any of the embodiments 3 to 24, wherein L represents a C_{1-4}alkylene chain, optionally substituted, by one or more C_{1-6} alkyl, preferably, two methyl groups.

26. The method according to any of the embodiments 3 to 25, wherein R1 represents hydrogen or C_{2-22}acyl, such as. hydrogen or C_{2-6}acyl.

27. The method according to any of the embodiments 3 to 26, wherein R2 represents hydrogen or C_{2-22} acyl, such as C_{2-6}acyl.

28. The method according to any of the embodiments 3 to 27, wherein R3 represents hydroxy or C_{1-22}alkoxy such as C_{1-6}alkoxy.

29. The method according to any of the embodiments 3 to 28, wherein R4 represents hydrogen or C_{2-22} acyl such

as C_{2-6}acyl.

30. The method according to any of the embodiments 3 to 29, wherein R5 represents hydrogen or C_{2-22} acyl such as hydrogen or C_{2-6}acyl.

31. The method according to any of the embodiments 3 to 30, wherein R6 represents hydroxy or C_{1-22}alkoxy such as C_{1-6}alkoxy.

32. The method according to any of the embodiments 3 to 31, wherein L represents methylene or ethylene.

33. The method according to any of the embodiments 3 to 32, wherein the cysteine/cystine analogs comprises one or more compounds selected from the compounds represented by formula 1, and salts thereof, wherein L, R1, R2, R3 and R7 areas defined above,
with the provisos

(i) that if one of the R1 or R2 groups, represents, C_{6-22}acyl, C_{6-22}alkyl which group is optionally substituted by one or two substituents selected from hydroxy and C_{1-22}alkoxy; C_{6-22} heteroalkyl, hydroxysulphonyl, C_{6-22}alkylsulphonyl, or C_{5-22}aryl sulphonyl; then
the remaining R1 or R2 group represents independently hydrogen, amino carbonyl, C_{2-6}acyl, C_{1-6}alkyl which group is optionally substituted by one or two substituents selected from hydroxy and C_{1-6}alkoxy; C_{1-6} heteroalkyl, hydroxysulphonyl, C_{1-6}alkylsulphonyl, or C_{5-10}aryl sulphonyl; and
R3 represents hydroxy; NH_2; C_{1-6}alkoxy wherein one or more carbons of the C_{1-22} alkyl may be optionally replaced by an aryl or an heteroaryl; C_{1-6} alkylamino which group is optionally substituted by a hydroxy or a C_{1-6} alkyl; hydroxy amino; C_{1-6} alkoxy amino; C_{1-6} alkylamino wherein one or more carbons of the C_{1-6}alkyl are replaced by an aryl or an heteroaryl; C_{1-6}heteroalkylamino; di(C_{1-6} alkyl)amino which is optionally substituted by a hydroxy; di(C_{1-6}alkyl)amino wherein one or more carbons of the C_{1-6}alkyl are replaced by an aryl or heteroaryl; or di(C_{1-6}heteroalkyl)amino; and
(ii) that if
R3 represents C_{6-22}alkoxy wherein one or more carbons of the C_{6-22} alkyl may be optionally replaced by an aryl or an heteroaryl; C_{6-22} alkylamino which group is optionally substituted by a hydroxy or a C_{1-22} alkyl; hydroxy amino; C_{6-22} alkoxy amino; C_{6-22} alkylamino wherein one or more carbons of the C_{1-22}alkyl are replaced by an aryl or an heteroaryl; C_{6-22}heteroalkylamino; di(C_{6-22} alkyl)amino which is optionally substituted by a hydroxy; di(C_{6-22}alkyl)amino wherein one or more carbons of the C_{6-22}alkyl are replaced by an aryl or heteroaryl; or di(C_{6-22}heteroalkyl)amino,
then R1 and R2 independently represents hydrogen, amino carbonyl, C_{2-6}acyl, C_{1-6}alkyl which group is optionally substituted by one or two substituents selected from hydroxy and C_{1-6}alkoxy; C_{1-6} heteroalkyl, hydroxysulphonyl, C_{1-6}alkylsulphonyl, or C_{5-10}aryl sulphonyl.

34. The method according to any of the embodiments 3 to 33, wherein the cysteine/cystine analogs comprise or consist of the compound represented by formula 2, and salts thereof, wherein L, R1, R2, R3, R4, R5, R6 and R7 are as defined above, with the provisos:

(i) that if
one of the of R1 andR2 groups represents C_{6-22}acyl, C_{6-22}alkyl which group is optionally substituted by one or two substituents selected from hydroxy and C_{1-22}alkoxy; C_{6-22} heteroalkyl, hydroxysulphonyl, C_{6-22}alkylsulphonyl, or C_{5-22}aryl sulphonyl; and if one of the R4 and R5 groups represents C_{6-22}acyl, C_{6-22}alkyl which group is optionally substituted by one or two substituents selected from hydroxy and C_{1-22}alkoxy; C_{6-22} heteroalkyl, hydroxysulphonyl, C_{6-22}alkylsulphonyl, or C_{5-22}aryl sulphonyl, then
the remaining R1, R2, R4, or R5 groups independently represent hydrogen, amino carbonyl, C_{2-6}acyl, C_{1-6}alkyl which group is optionally substituted by one or two substituents selected from hydroxy and C_{1-6}alkoxy; C_{1-6} heteroalkyl, hydroxysulphonyl, C_{1-6}alkylsulphonyl, or C_{5-10}aryl sulphonyl; and
R3 and R6 independently represents hydroxy; NH_2; C_{1-6}alkoxy wherein one or more carbons of the C_{1-22} alkyl may be optionally replaced by an aryl or an heteroaryl; C_{1-6} alkylamino which group is optionally substituted by a hydroxy or a C_{1-6} alkyl; hydroxy amino; C_{1-6} alkoxy amino; C_{1-6} alkylamino wherein one or more carbons of the C_{1-6}alkyl are replaced by an aryl or an heteroaryl; C_{1-6}heteroalkylamino; di(C_{1-6} alkyl)amino which is optionally substituted by a hydroxy; di(C_{1-6}alkyl)amino wherein one or more carbons of the C_{1-6}alkyl are replaced by an aryl or heteroaryl; or di(C_{1-6}heteroalkyl)amino;
(ii) that if only if one of R1, R2, R4 and R5 groups independently represent C_{6-22}acyl, C_{6-22}alkyl which group is optionally substituted by one or two substituents selected from hydroxy and C_{1-22}alkoxy; C_{6-22} heteroalkyl, hydroxysulphonyl, C_{6-22}alkylsulphonyl, or C_{5-22}aryl sulphonyl; then
one of R3 and R6 may r represent C_{6-22}alkoxy wherein one or more carbons of the C_{1-22} alkyl may be optionally replaced by an aryl or an heteroaryl; C_{6-22} alkylamino which group is optionally substituted by a hydroxy or a

C_{1-22} alkyl; C_{6-22} alkoxy amino; C_{6-22} alkylamino wherein one or more carbons of the C_{1-22}alkyl are replaced by an aryl or an heteroaryl; C_{6-22}heteroalkylamino; di(C_{6-22} alkyl)amino which is optionally substituted by a hydroxy; di(C_{6-22}alkyl)amino wherein one or more carbons of the C_{6-22}alkyl are replaced by an aryl or heteroaryl; or di(C_{6-22}heteroalkyl)amino;

(iii) that if none of the R1, R2, R4 and R5 groups represents C_{6-22}acyl, C_{6-22}alkyl which group is optionally substituted by one or two substituents selected from hydroxy and C_{1-22}alkoxy; C_{6-22} heteroalkyl, hydroxysulphonyl, C_{6-22}alkylsulphonyl, or C_{5-22}aryl sulphonyl; then
R3 and R6 may both represent independently C_{6-22}alkoxy wherein one or more carbons of the C_{1-22} alkyl may be optionally replaced by an aryl or an heteroaryl; C_{6-22} alkylamino which group is optionally substituted by a hydroxy or a C_{1-22} alkyl; C_{6-22} alkoxy amino; C_{6-22} alkylamino wherein one or more carbons of the C_{1-22}alkyl are replaced by an aryl or an heteroaryl; C_{6-22}heteroalkylamino; di(C_{6-22} alkyl)amino which is optionally substituted by a hydroxy; di(C_{6-22}alkyl)amino wherein one or more carbons of the C_{6-22}alkyl are replaced by an aryl or heteroaryl; or di(C_{6-22}heteroalkyl)amino.

35. The method according to any of the embodiments 3 to 34, wherein the cysteine/cystine analogs comprise or consist of salts of formula 1 and 2.

36. The method according the embodiment 35, wherein the salts are formed by reaction of a hydroxy group present on the cysteine/cystine analogs.

37. The method according the embodiments 35 or 36, wherein the salts include alkali metal salts, for example sodium, potassium or lithium salts; alkali earth metal salts, for example magnesium or calcium salts; ammonium salts, for example tetra alkyl or aryl ammonium salts; sulphonium salts, for example trialkyl or aryl sulphonium; and phosphonium salts, for example tetra alkyl or aryl phosphonium salts.

38. The method according any one embodiments 35 to 37, wherein the salts are formed by reaction of an amino group present on the cysteine/cysteine analogs, such as the reaction of the amino group with an inorganic acid or an organic acid and include mono- or di- HCl salts, H_2SO_4 salts, H_3PO_4 salts, acetate and fumarate.

39. The method according any one embodiments 3 to 38, wherein the cysteine/cystine analogs as defined here above have the same chirality as L-cysteine.

40. The method according any one embodiments 3 to 39, wherein the cysteine/cysteine analogs are selected from N,N'-diacetyl-L-cystine-dimethylester, N-Acetyl-L-cysteine and N,N'-Diacetyl-L-cystine, or S-sulfocysteine.

41. The method according to any one of embodiments 3 to 40, wherein the cysteine/cystine analogs comprise, or consist of, compounds that have the same chirality as L-cysteine.

42. The method according to any one of embodiments 3 to 41, wherein the cysteine/cystine analogs consists of, N,N'-diacetyl-L-cystine-dimethylester.

43. The method according to any one of the preceding embodiments, wherein said cell culture medium comprises:

(a) cysteine/cystine analogs; and

(b) cysteine and/or cystine,

wherein the molar ratio of (a) to (b) is between 1:18 and 18:1.

44. The method according to any one of the preceding embodiments, wherein the method comprises the steps of:

(i) inoculating said host cells in a basal medium, wherein the basal medium optionally comprises an initial amount of:

(a) cysteine/cystine analogs; and/or
(b) cysteine and/or cystine,

(ii) progressing the culture through a production phase wherein the recombinant protein is produced by the cells, wherein, during said production phase, the cell culture medium is supplemented with:

(a) cysteine/cystine analogs; and/or
(b) cysteine and/or cystine,

wherein (a) and (b) may be added simultaneously or sequentially,
wherein, when the contents of the basal medium and the total supplements added are added up, the molar ratio of (a) to (b) is between 1:18 and 18:1.

45. The method according to embodiment 43 or 44, wherein said molar ratio of (a) to (b) is between 1:15 and 15:1,

e.g. between 1:12 and 12:1, such as between 1:10 and 10:1, e.g. between 1:8 and 8:1, such as between 1:6 and 6:1, e.g. between 1:4 and 4:1, such as between 1:3 and 3:1, e.g. between 1:2 and 2:1, such as between 1.5:1 and 1:1.5, e.g. a molar ratio of 1:1.

46. The method according to embodiment 43 or 44, wherein the concentration of (b) in said basal medium is equivalent to between 0.05 and 5 mmol/L of cysteine, such as between 0.1 and 1 mmol/L, e.g. between 0.2 and 0.6 mmol/L.

47. The method according to any one of embodiments 43 to 46, wherein, during the production phase, the medium is supplemented with (a) cysteine/cystine analogs and (b) cysteine and/or cystine, wherein the sum of (a) and (b) added to the culture over the entire production phase is equivalent to between 1 and 75 mmol/L of cysteine, such as between 2 and 20 mmol/L, e.g. between 4 and 10 mmol/L.

48. The method according to any one of embodiments 43 to 46, wherein, during the production phase, the medium is supplemented daily with (a) cysteine/cystine analogs and (b) cysteine and/or cystine, wherein the daily addition brings the concentration of (a) + (b) to a concentration equivalent to between 0.05 and 5 mmol/L of cysteine, such as between 0.1 and 1 mmol/L.

49. The method according to any one of embodiments 43 to 48, wherein, during said production phase, the cell culture medium is supplemented with:

- cysteine or cystine up to a total amount of from 10 wt% to 30 wt% of the expected total amount of recombinant protein produced; and/or
- tryptophan up to a total amount of from 8 wt% to 35 wt% of the expected total amount of recombinant protein produced.

50. The method according to any one of embodiments 43 to 49, wherein

- the cysteine or cystine concentration in the cell culture does not exceed 0.9 g/L at any time point during the production phase, preferably wherein the cysteine or cystine concentration in the cell culture does not exceed 0.3 g/L at any time point during the production phase, and/or
- the tryptophan concentration in the cell culture does not exceed 0.6 g/L at any time point during the production phase, preferably wherein the tryptophan concentration in the cell culture does not exceed 0.3 g/L at any time point during the production phase.

51. The method according to any one of embodiments 43 to 50, wherein

- the total amount of cysteine or cystine provided during the process is from 2.9 to 12 g/(10^{12} cells), such as from 2.9 to 7 g/(10^{12} cells), wherein cells refers to the expected integral viable cell count at the end of the production phase, and/or
- the total amount of tryptophan provided during the process is from 2.5 to 7 g/(10^{12} cells), such as from 2.5 to 3.5 g/(10^{12} cells/L), wherein cells refers to the expected integral viable cell count at the end of the production phase.

52. The method according to any one of the preceding embodiments, wherein the method is a batch method.

53. The method according to any one of the preceding embodiments, wherein the recombinant protein is produced during a production phase, preferably wherein the production phase has a duration of at least 7 days, more preferably at least 14 days.

54. The method according to any one of the preceding embodiments, wherein the culture is supplemented daily in the production phase.

55. The method according to any one of the preceding embodiments, wherein the production phase is carried out in a bioreactor, preferably with a volume of equal or more than 50 L, equal or more than 100 L, equal or more than 500 L, equal or more than 1000 L, equal or more than 2,000 L, equal or more than 5,000 L, equal or more than 10,000 L or equal or more than 20,000 L.

56. The method according to any one of the preceding embodiments, wherein the host cells are mammalian cells, preferably CHO cells.

57. The method according to any one of the preceding embodiments, wherein the recombinant protein is an antibody or an antigen-binding fragment thereof.

58. The method according to embodiment 57, wherein the antibody or antigen-binding fragment thereof is:

i) an antibody or antigen-binding fragment thereof which

a. comprises CDR-H1 having the sequence as defined in SEQ ID NO:1; CDR-H2 having the sequence as defined in SEQ ID NO:2; CDR-H3 having the sequence as defined in SEQ ID NO:3; CDR-L1 having the sequence as defined in SEQ ID NO:4; CDR-L2 having the sequence as defined in SEQ ID NO:5 and CDR-L3 having the sequence as defined in SEQ ID NO:6; or
b. comprises a light variable region having the sequence as defined in SEQ ID NO: 7 and a heavy variable region having the sequence as defined in SEQ ID NO: 8; or
c. comprises a light variable region having at least 80% identity or similarity, preferably 90% identity or similarity to the sequence as defined in SEQ ID NO: 7 and a heavy variable region having at least 80% identity or similarity, preferably 90% identity or similarity to the sequence as defined in SEQ ID NO: 8;
d. comprises a light variable region having the sequence as defined in SEQ ID NO: 7 and a heavy chain having the sequence as defined in SEQ ID NO: 11; or
e. comprises a light variable region having at least 80% identity or similarity, preferably 90% identity or similarity to the sequence as defined in SEQ ID NO: 7 and a heavy chain having at least 80% identity or similarity, preferably 90% identity or similarity to the sequence as defined in SEQ ID NO: 11; or

ii) an antibody which comprises a light chain having the sequence as defined in SEQ ID NO: 9 and a heavy chain having the sequence as defined in SEQ ID NO: 10; or
iii) an antibody which comprises a light chain having at least 80% identity or similarity, preferably 90% identity or similarity to the sequence as defined in SEQ ID NO: 9 and a heavy chain having at least 80% identity or similarity, preferably 90% identity or similarity to the sequence as defined in SEQ ID NO: 10.

59. The method according to any one of the preceding embodiments, wherein the recombinant protein is a protein which is not colorless at a concentration of 10 mg/ml or more, such as 50 mg/ml or more, when produced by host cells grown in a cell culture medium not comprising cysteine/cystine analogs, wherein color e.g. is determined as described in the Examples herein.
60. The method according to any one of the preceding embodiments, wherein the method comprises the step of recovering the recombinant protein from the cell culture medium and a further step of purifying the recombinant protein.
61. The method according to embodiment 60 wherein the purification comprises Protein A chromatography.
62. The method according to embodiment 60 or 61, further comprising the step of formulating the purified recombinant protein.
63. A method for producing a recombinant protein preparation comprising:

(i) inoculating said host cells in a basal medium, wherein the basal medium optionally comprises an initial amount of:

(a) cysteine/cystine analogs; and/or

(b) cysteine and/or cystine,

(ii) progressing the culture through a production phase wherein the recombinant protein is produced by the cells, wherein, during said production phase, the cell culture medium is supplemented with:

(a) cysteine/cystine analogs; and/or

(b) cysteine and/or cystine,

wherein (a) and (b) may be added simultaneously or sequentially,
wherein, when the contents of the basal medium and the total supplements added are added up, the molar ratio of (a) to (b) is between 1:18 and 18:1.
64. The method according to embodiment 39, wherein the cysteine/cystine analogs are as described in embodiments 3 - 41 above.
65. The method according embodiments 63 or 64, wherein said molar ratio of (a) to (b) is between 1:15 and 15:1, e.g. between 1:12 and 12:1, such as between 1:10 and 10:1, e.g. between 1:8 and 8:1, such as between 1:6 and 6:1, e.g. between 1:4 and 4:1, such as between 1:3 and 3:1, e.g. between 1:2 and 2:1, such as between 1.5:1 and 1:1.5, e.g. a molar ratio of 1:1.

66. The method according to any one of embodiments 63 to 65, wherein the concentration of (b) in said basal medium is equivalent to between 0.05 and 5 mmol/L of cysteine, such as between 0.1 and 1 mmol/L, e.g. between 0.2 and 0.6 mmol/L.

67. The method according to any one of embodiments 63 to 66, wherein, during the production phase, the medium is supplemented with (a) cysteine/cystine analogs and (b) cysteine and/or cystine, wherein the sum of (a) and (b) added to the culture over the entire production phase is equivalent to between 1 and 75 mmol/L of cysteine, such as between 2 and 20 mmol/L, e.g. between 4 and 10 mmol/L.

68. The method according to any one of embodiments 63 to 67, wherein, during the production phase, the medium is supplemented daily with (a) cysteine/cystine analogs and (b) cysteine and/or cystine, wherein the daily addition brings the concentration of (a) + (b) to a concentration equivalent to between 0.05 and 5 mmol/L of cysteine, such as between 0.1 and 1 mmol/L.

69. The method according to any one of embodiments 63 to 68, wherein, during said production phase, the cell culture medium is supplemented with:

- cysteine or cystine up to a total amount of from 10 wt% to 30 wt% of the expected total amount of recombinant protein produced; and/or
- tryptophan up to a total amount of from 8 wt% to 35 wt% of the expected total amount of recombinant protein produced.

70. The method according to any one of embodiments 63 to 69, wherein

- the cysteine or cystine concentration in the cell culture does not exceed 0.9 g/L at any time point during the production phase, preferably wherein the cysteine or cystine concentration in the cell culture does not exceed 0.3 g/L at any time point during the production phase, and/or
- the tryptophan concentration in the cell culture does not exceed 0.6 g/L at any time point during the production phase, preferably wherein the tryptophan concentration in the cell culture does not exceed 0.3 g/L at any time point during the production phase.

71. The method according to any one of embodiments 63 to 70, wherein

- the total amount of cysteine or cystine provided during the process is from 2.9 to 12 g/(10^{12} cells), such as from 2.9 to 7 g/(10^{12} cells), wherein cells refers to the expected integral viable cell count at the end of the production phase, and/or
- the total amount of tryptophan provided during the process is from 2.5 to 7 g/(10^{12} cells), such as from 2.5 to 3.5 g/(10^{12} cells/L), wherein cells refers to the expected integral viable cell count at the end of the production phase.

72. The method according to any one of embodiments 63 to 71, wherein the method is a batch method.

73. The method according to any one of embodiments 63 to 72, wherein the recombinant protein is produced during a production phase, preferably wherein the production phase has a duration of at least 7 days, more preferably at least 14 days.

74. The method according to any one of embodiments 63 to 73, wherein the culture is supplemented daily in the production phase.

75. The method according to any one of embodiments 63 to 74, wherein the production phase is carried out in a bioreactor, preferably with a volume of equal or more than 50 L, equal or more than 100 L, equal or more than 500 L, equal or more than 1000 L, equal or more than 2,000 L, equal or more than 5,000 L, equal or more than 10,000 L or equal or more than 20,000 L.

76. The method according to any one of embodiments 63 to 75, wherein the host cells are mammalian cells, preferably CHO cells.

77. The method according to any one any one of embodiments 63 to 76, wherein the recombinant protein is an antibody or an antigen-binding fragment thereof.

78. The method according to embodiment 77, wherein the antibody or antigen-binding fragment thereof is:

i) an antibody or antigen-binding fragment thereof which

a. comprises CDR-H1 having the sequence as defined in SEQ ID NO:1; CDR-H2 having the sequence as

defined in SEQ ID NO:2; CDR-H3 having the sequence as defined in SEQ ID NO:3; CDR-L1 having the sequence as defined in SEQ ID NO:4; CDR-L2 having the sequence as defined in SEQ ID NO:5 and CDR-L3 having the sequence as defined in SEQ ID NO:6; or

b. comprises a light variable region having the sequence as defined in SEQ ID NO: 7 and a heavy variable region having the sequence as defined in SEQ ID NO: 8; or

c. comprises a light variable region having at least 80% identity or similarity, preferably 90% identity or similarity to the sequence as defined in SEQ ID NO: 7 and a heavy variable region having at least 80% identity or similarity, preferably 90% identity or similarity to the sequence as defined in SEQ ID NO: 8;

d. comprises a light variable region having the sequence as defined in SEQ ID NO: 7 and a heavy chain having the sequence as defined in SEQ ID NO: 11; or

e. comprises a light variable region having at least 80% identity or similarity, preferably 90% identity or similarity to the sequence as defined in SEQ ID NO: 7 and a heavy chain having at least 80% identity or similarity, preferably 90% identity or similarity to the sequence as defined in SEQ ID NO: 11; or

ii) an antibody which comprises a light chain having the sequence as defined in SEQ ID NO: 9 and a heavy chain having the sequence as defined in SEQ ID NO: 10; or

iii) an antibody which comprises a light chain having at least 80% identity or similarity, preferably 90% identity or similarity to the sequence as defined in SEQ ID NO: 9 and a heavy chain having at least 80% identity or similarity, preferably 90% identity or similarity to the sequence as defined in SEQ ID NO: 10.

79. The method according to any one of embodiments 63 to 78, wherein the recombinant protein is a protein which is not colorless at a concentration of 10 mg/ml or more, such as 50 mg/ml or more, when produced by host cells grown in a cell culture medium not comprising cysteine/cystine analogs, wherein color e.g. is determined as described in the Examples herein.

80. The method according to any one of embodiments 63 to 79, wherein the method comprises the step of recovering the recombinant protein from the cell culture medium and a further step of purifying the recombinant protein.

81. The method according to embodiment 80 wherein the purification comprises Protein A chromatography.

82. The method according to embodiment 80 or 81, further comprising the step of formulating the purified recombinant protein.

83. The method according to any one of embodiments 63 to 82, wherein said host cells are CHO cells and said cysteine/cystine analog is N,N'-diacetyl-L-cystine-dimethylester.

84. A recombinant protein preparation obtainable or obtained by the method according to any one of the preceding embodiments.

85. A cell culture medium suitable for culturing mammalian cells comprising N,N'-diacetyl-L-cystine-dimethylester.

[0102] The invention will now be further described by way of examples with references to embodiments illustrated in the accompanying drawings.

EXAMPLES

Abbreviations

[0103] mAb: monoclonal antibody; Cys: cysteine or cystine; APG: acidic peak group; VCC: viable cell count; DO: dissolved oxygen; CO_2: carbon dioxide; CHO: Chinese Hamster Ovary; UPLC: Ultra Performance Liquid Chromatography.

Example 1

[0104] The ability of cysteine/cystine derivatives to reduce color and acidic species of an antibody preparation produced recombinantly by host cells grown in cell culture was evaluated. For this experiment, a scale-down model in shake flask was used. CHO-DG44 cells producing a full-length antibody, mAb1, were inoculated in 100mL of basal media at a seeding density of 0.35×10^6 cells/mL mAb1 comprises a light chain having the sequence as defined in SEQ ID NO: 9 and a heavy chain having the sequence as defined in SEQ ID NO: 10.

[0105] The shake flask vessels were 250 mL shake flasks (corning) disposed in an agitated incubator (Infors) protected from light. The cells were cultivated for 14 days at 36.8°C with 80% humidity. The cell cultures were agitated at 140 rpm at the beginning of the process. The agitation was increased during the process progressively to reach 250 rpm to maintain a DO% at 40% in the shake flask, as is it done in a 2L bioreactor process. The cell culture was maintained at pH 7.0 ± 0.2 by decreasing the CO_2 in the incubator from 5% before the process to 2% during the process. Cells were

cultured in a fed-batch mode with bolus addition of Feed every day of culture. The basal media contained 0.04 mmol/L of cysteine + 0.38 mmol/L of cystine. The Feed contained 153.3 mmol/L of cysteine. Over the entire 14-day production phase, the total volume of Feed added corresponded to 4.47% (v/v) of the culture start volume.

[0106] The cysteine present in the Feed was replaced by different cysteine and cystine derivatives at different percentages as presented in Table 1. Osmolality was daily monitored using an osmometer from Advanced Instruments. Off line pH, dissolved O_2 and dissolved CO_2 were daily monitored using a model BioProfile pHOx® blood gas analyser (Nova Biomedical Corporation, Waltham, MA). Metabolites concentrations were daily determined using a CedexBioHT system (Roche). Viable cell concentration and cell viability were measured daily using a ViCell automated cell counter (Beckman Coulter). The cell culture fluid was collected daily by centrifuging 1mL of cell culture fluid for determination of antibody titer using protein A HPLC (Waters). At the end of the 14 days of culture, the cell culture fluid was harvested by centrifugation. The supernatant was then purified using a protein-A affinity chromatography (MabSelect Sure, GE). The protein A eluates were used for the molecular weight determination using UPLC size exclusion (Waters).

[0107] The relative percentage of main, acidic (APG for Acidic Peak Group) and basic (BPG for Basic Peak Group) isoforms of the purified mAb was determined by Imaged Capillary Electrophoresis (ProteinSimple iCE3). Another part of the protein A eluates was concentrated to 40mg/mL using Amicon centricon centrifugal filter devices (Millipore).

[0108] Color intensity of the concentrated antibody composition was measured in the concentrated protein A eluates using a spectrophotometer by transmission (UltrascanPro) and compared to the CIE (commission internationale de l'éclairage) scale. The numerical results were normalized to the concentration of 40mg/mL.

[0109] Figure 1 shows the profile of cell growth using different cysteine and cystine derivatives with different replacement ratio of the cysteine in the Feed. In conditions with 100% of Cys derivative, cell growth slowed down from day 6 compared to the control condition, except for the 100% S-sulfocysteine condition where cells rapidly died on day 7. The viable cell concentration was similar to the control condition for the 50% derivative feeds until day 10. From day 10, the viable cell densities were higher than the control condition. However, for 50% S-sulfocysteine, the viable cell concentration reached a maximum of $2x10^6$ cells/mL, significantly lower than the control condition. Cells did not grow as well as the control when 100% of Cys was replaced with a Cys derivative suggesting that cysteine derivatives cannot fully replace cysteine. On the other hand, the condition with 50% derivatives exhibited a better growth than the control condition suggesting a positive impact of the cysteine/cystine derivatives when a minimum of cysteine is present. S-sulfocysteine does not seem to act as the other derivatives tested with respect to enabling cell growth.

[0110] In view of the cell death observed with the 100% cysteine derivatives, other performance and product quality results were analyzed only for 50% cysteine derivative conditions.

[0111] The product titer was higher in cultures using 50% of cysteine derivatives than the control condition except when the derivative used was S-sulfocysteine (Figure 2).

The b*value of the concentrated protein-A eluate was decreased of between 6.5% to 21.2% compared to control condition, with the best result observed with 50% of N,N'-diacetyl-L-cystine dimethylester (CAS Registry number 32381-28-5, e.g. obtainable from Bachem AG) (Figure 3). Likewise, a decrease on acidic variants level was also observed of between 7.6% to 24.8% compared to control condition, with the best result observed with 50% of N,N'-diacetyl-L-cystine dimethylester (Figure 4). This decrease in acidic species level correlated with an increase of main charge species (Figure 5) with no significant increase of basic variant level, except for the S-sulfocysteine (not shown). These results confirmed the reduction of the micro-heterogeneity of the recombinant protein.

Table 1 : Different compositions of Feed tested

Derivatives used	% of cysteine replaced by the derivatives in Feed (molar equivalence)
N-Acetyl-L-cysteine	100
	50
N,N'-Diacetyl-L-cystine	100
	50
N,N'-diacetyl-L-cystine dimethylester	100
	50
S-Sulfocysteine	100
	50

Example 2

[0112] The ability of cysteine/cystine derivatives to reduce color and acidic species of a recombinant protein preparation in cell culture was evaluated using another antibody-producing cell line, cell line 2. For this experiment a scale-down model in shake flask was used. CHO-DG44 cells producing amultispecific antibody derivative were inoculated in 100mL of basal media at a seeding density of $0.35x10^6$ cells/mL. The shake flask vessels were 250 mL shake flasks (corning) disposed in an agitated incubator (Infors) protected from light. The cells were cultivated for 14 days at 36.8°C with 80% of humidity. The cell cultures were agitated at 140 rpm at the beginning of the process. The agitation was increased during the process progressively to reach 250 rpm to maintain a DO% at 40% in the shake flask, as is it done in the 2L bioreactor process. The cell culture was maintained at pH 7.0 ± 0.2 by decreasing the CO_2 in the incubator from 5% the process to 2% during the process. Cells were cultured in a fed-batch mode with bolus addition of Feed every day of culture. The basal media contained 0.04 mmol/L of cysteine + 0.38 mmol/L of cystine. The Feed contained 153.3 mmol/L of cysteine. Over the entire 14-day production phase, the total volume of Feed added corresponded to 2.81% (v/v) of the culture start volume.

[0113] The cysteine present in the Feed was replaced by different cysteine and cystine derivatives with a replacement ratio of 50% equimolar (Table 1). A condition with only 50% of the control cysteine levels was also added to test whether the improvements observed were due to the presence of the cysteine derivative or to the reduction of cysteine only. Osmolality was daily monitored using an osmometer from Advanced instrument. Off line pH, dissolved O_2 and dissolved CO_2 were daily monitored using a Nova Biomedical Phox (Nova biomedical). Metabolites concentrations were daily determined using a CedexBioHT system (Roche).Viable cell concentration and cell viability were measured daily using a ViCell automated cell counter (Beckman Coulter). The cell culture fluid was collected daily by centrifuging 1mL of cell culture fluid for determination of antibody titer using protein L Octet measurement (Pall). At the end of the 14 days of culture, the cell culture fluid was harvested by centrifugation. The supernatant was then purified using a protein L affinity chromatography (CaptoL, GE). The protein L eluates were used for the molecular weight determination using UPLC size exclusion (Waters) and for the charge variants determination using isocapillary focusing (ProteinSimple iCE3). The recombinant protein produced with cell line 2 was not a colored molecule. Therefore, no color measurement was performed in this case.

[0114] Figure 6 shows the profile of cell growth using different cysteine and cystine derivatives with a replacement ratio of 50% of the cysteine in the Feed. Similar cell growth profiles were observed between control condition and conditions with cysteine and cystine derivatives. Thus, these components do not impact the cell growth for this cell line. The product titer was also similar to the control condition for all cysteine and cystine derivatives (Figure 7).

[0115] As can be seen in Figure 8, a decrease on acidic variants level was observed of between 46.6% to 50.2% compared to control condition with the two cystine derivatives, N,N'-diacetyl-L-cystine and N,N'-diacetyl-L-cystine dimethylester. A decrease of 14.5% of acidic variant level was observed with N-acetyl-cysteine. This decrease in acidic species level correlated with an increase of main charge species (Figure 9) with no significant increase of basic variant level (data not shown). These results confirmed the reduction of the micro-heterogeneity of the recombinant protein by cystine derivative. On the other hand, an increase between 5% and 10% of acidic species was observed with S-sulfocysteine and with the reduction of cysteine only.

Example 3

[0116] The ability of cysteine/cystine derivatives to reduce color and acidic species of a recombinant protein preparation produced in cell culture was evaluated in a bioreactor for two cell lines. This model is more representative of the large-scale production than the shake flask due to the geometry and additional control. CHO-DG44 cells from cell line 1 and 2 were inoculated in 1300mL of basal media at a seeding density of $0.35x10^6$ cells /mL in 2L stirred bioreactor (Sartorius). The cells were cultivated during 14 days in fed-batch mode. The cell cultures were agitated at 280 rpm and the DO was maintained at 40%. The cell culture was maintained at pH 7.0 ± 0.2 with an automatic CO_2 sparging regulation. Cells were cultured in a fed-batch mode with bolus addition of Feed every day of culture. The basal media contained 0.04 mmol/L of cysteine + 0.38 mmol/L of cystine. The Feed contained 153.3 mmol/L of cysteine. Over the entire 14-day production phase, the total volume of Feed added corresponded to 5.7% (w/w) of the culture start volume for cell line 1 and to 3.1% (w/w) of the culture start volume for cell line 2.

[0117] The cysteine present in the Feed was replaced by different cysteine- and cystine derivatives at different percentages as presented in Table 2. A condition with only 50% of cysteine was added to test whether the improvements observed were due to the cysteine derivative or to the reduction of cysteine only. Osmolality was daily monitored using an osmometer from Advanced instrument. Off line pH, dissolved O_2 and dissolved CO_2 were daily monitored using a Nova Biomedical Phox (Nova biomedical). Metabolites concentrations were daily determined using a CedexBioHT system (Roche). Viable cell concentration and cell viability were measured daily using a ViCell automated cell counter (Beckman Coulter). The cell culture fluid was collected daily by centrifuging 1mL of cell culture fluid for determination of antibody

titer by HPLC prot A (waters) or using protein-L Octet measurement (Pall). At the end of the 14 days of culture, the cell culture fluid was harvested by centrifugation. The supernatant was then purified using a protein-A or L affinity chromatography (GE). The protein-A or L eluates were used for the molecular weight determination using UPLC size exclusion (Waters) and for the charge variants determination using isocapillary focusing (ProteinSimple iCE3). Another part of the protein-A eluates (only from cell line 1) was concentrated to 40mg/mL using Amicon centricon centrifugal filter devices (Millipore). Color intensity of the concentrated antibody composition was measured in the concentrated protein A eluates using a spectrophotometer by transmission (UltrascanPro) and compared to the CIE (commission internationale de l'éclairage) scale. The numerical results were normalized to the concentration of 40mg/mL.

Table 2: Different compositions of Feed tested with the cell line 1 and 2 in 2L bioreactor

Derivatives used	% of cysteine replaced by the derivatives in Feed (molar equivalence)
N-Acetyl-L-cysteine	50
N,N'-Diacetyl-L-cystine	50
N,N'-diacetyl-L-cystine dimethylester	50

Cell line 1

[0118] Similar cell growth trends were observed for all conditions tested (Figure 10).

[0119] A significant decrease of antibody titer was observed with N,N'-diacetyl-cystine and N-acetyl-cysteine between 10% and 17% but a non-significant decrease of less than 5% was observed with N,N'-diacetyl-L-cystine dimethylester (Figure 11). A significant decrease of titer was observed also with the 50% cysteine feed only, suggesting that N,N'-diacetyl-L-cystine dimethylester improves the titer.

[0120] Regarding acidic species level, a decrease of 5% with N,N'-diacetyl-L-Cystine, 10% with N-acetyl-L-cysteine and 15% with N,N'-diacetyl-L-cystine dimethylester was observed (Figure 12). This reduction correlated with an increase of main species (Figure 13). A similar impact on charge variant was observed at 2L scale as in shake flasks. This reduction was also observed with 50% cysteine feed only. This suggest that the acidic species level reduction here may be due to the reduction of cysteine. Decreases of the b value level of 2.5% with N,N'-diacetyl-L-Cystine, 13% with N-acetyl-L-cysteine and 25% with N,N'-diacetyl-L-cystine dimethylester were observed. A similar impact on product color intensity was observed at 2L scale as in shake flasks. No reduction of the b*value was observed with the 50% cysteine feed only. The impact of N,N'-diacetyl-L-cystine dimethylester on coloration was confirmed.

Cell line 2

[0121] Similar cell growth trends were observed for all conditions tested (Figure 15).

[0122] A significant increase of antibody titer of between 15% to 23% was observed with N,N'-diacetyl-cystine, N-acetyl-cysteine and N,N'-diacetyl-L-cystine dimethylester (Figure 16). This result is thus different from the results obtained in shake flasks.

[0123] Regarding acidic species level, a decrease of 8% with N,N'-diacetyl-L-Cystine, 13% with N-acetyl-L-cysteine and 13% with N,N'-diacetyl-L-cystine dimethylester was observed (Figure 17). This reduction correlated with an increase of main species (Figure 18). A similar trend for charge variants was observed at 2L scale compared to shake flasks, except for N-acetyl-cysteine. The impact of the replacement of cysteine by N,N'-diacetyl-L-cystine dimethylester on charge variants was confirmed.

[0124] Decrease of 27% of the acid species level was observed with 50% cysteine feed. This indicates that the reduction of cysteine is the main driver for the reduction of acidic species. A titer increase of 10% was observed with 50% cysteine feed instead of 15% to 23% with the cysteine/ cystine derivatives. The replacement of 50% of the cysteine by a cysteine / cystine derivative is a good compromise between titer increase and heterogeneity reduction for this cell line.

Example 4

[0125] The reproducibility of the results was evaluated in bioreactor with cell line 1. The experiments of Example 3 were repeated with an additional control and a bioreactor with 50% S-sulfocysteine 50% Cysteine Feed. The data from the two experiments were analyzed to confirm the effect previously observed with cysteine/cystine derivatives.

Table 3: Data used for the analysis

Feed	Number of replicates	
	Data set 1	Data set 2
Control 100% Cysteine Feed	1	2
50% Cysteine Feed	1	1
50% N-Acetyl-L-cysteine 50% Cysteine Feed	1	1
50% N,N'-Diacetyl-L-cystine 50% Cysteine Feed	1	1
50% N,N'-diacetyl-L-cystine dimethylester 50% Cysteine Feed	1	1
50% S-sulfocysteine 50% Cysteine Feed	0	1

[0126] No differences regarding cell growth were observed between day 0 and 8. From day 9 condition with S-sulfocysteine had a higher cell death than the control. 50% cysteine feed, 50% N,N'-diacetyl cystine 50% cysteine feed and 50% N-acetyl-cysteine 50% cysteine feed had higher VCCs than the control conditions. The 50% N,N'-diacetyl cystine dimethyl ester 50% cysteine feed condition was similar to the control conditions average (Figure 19).

[0127] A non significant decrease in antibody titer of less than 5% compared to the controls average was observed with N,N'-diacetyl cystine dimethyl ester. However, a decrease of 10% was observed for the 50% cysteine feed. The use of N,N'-diacetyl cysteine dimethyl ester has thus compensated 5% of the titer loss due to cysteine reduction in feed. Moreover, the higher VCC observed with 50% cysteine feed indicates an increase of the specific productivity with N,N'-diacetyl cystine dimethyl ester compared to 50% cysteine feed. This observation confirms that a loss of specific productivity due to reduction of cysteine can be compensated by addition of N,N'-diacetyl cystine dimethyl ester. A decrease of 10% was also observed with N-acetyl cysteine and N,N'-diacetyl cysteine. These molecules did not impact the productivity for this cell line. On the other hand, a decrease of 25% in titer was observed with S-sulfocysteine. This was due to the decrease of VCC and to the decrease of productivity due to the cysteine reduction, but not to a specific impact of S-sulfocysteine on the specific productivity (Figure 20).

[0128] Regarding acidic species level, an average decrease of 10% with N,N'-diacetyl-L-Cystine and N-acetyl-L-cysteine were observed (Figure 21). This reduction correlated with an increase of main species (Figure 22).

[0129] An average reduction of 15% was observed with N,N'-diacetyl-L-Cystine. This reduction is also observed with 50% cysteine feed only. This again suggests that the acidic species level reduction is due to the reduction of cysteine levels. Similar acidic species levels were observed between S-sulfocysteine and control condition. This suggest that S-sulfocysteine increased acidic species level normally decreased by a cysteine reduction.

[0130] Regarding b value level, an average decrease of 5% with S-sulfocysteine, 14% with N,N'-diacetyl-L-Cystine, 13% with N-acetyl-L-cysteine and 30% with N,N'-diacetyl-L-cystine dimethylesterwere observed. No reduction of the b*value was observed with the 50% cysteine feed only. The impact of N,N'-diacetyl-L-cystine dimethylester on coloration was confirmed (Figure 23).

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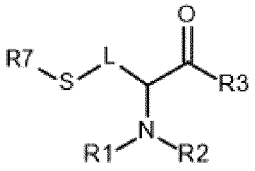
Leu	Val	Lys	Asp	Tyr	Phe	Pro	Glu	Pro	Val	Thr	Val	Ser	Trp	Asn	Ser
145					150					155					160
Gly	Ala	Leu	Thr	Ser	Gly	Val	His	Thr	Phe	Pro	Ala	Val	Leu	Gln	Ser
				165					170					175	
Ser	Gly	Leu	Tyr	Ser	Leu	Ser	Ser	Val	Val	Thr	Val	Pro	Ser	Ser	Ser
			180					185					190		
Leu	Gly	Thr	Gln	Thr	Tyr	Ile	Cys	Asn	Val	Asn	His	Lys	Pro	Ser	Asn
		195					200					205			
Thr	Lys	Val	Asp	Lys	Lys	Val	Glu	Pro	Lys	Ser	Cys				
	210					215					220				

Claims

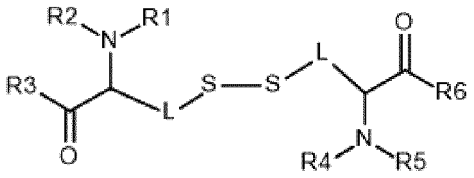
1. A method for reducing the heterogeneity of a population of recombinant proteins produced in cell culture, said method comprising growing host cells producing a recombinant protein in a cell culture medium wherein the cell culture medium comprises one or more cysteine/cystine analogs, wherein said reduction of heterogeneity is obtained without substantially reducing the titer of the recombinant protein at the end of the production process and wherein said reduction of heterogeneity comprises reducing:

   a. color or intensity of color;
   b. charge heterogeneity, preferably by reducing acidic peak group species (APG) and/or basic peak group species (BPG), whereby the main charge species does not decrease; and/or
   c. amino acid oxidation, preferably methionine oxidation.

2. The method according to claim 1, wherein the cysteine/cystine analogs comprise or consist of one or more compounds selected from the compounds represented by formula 1 and 2, and salts thereof:



1



2

wherein,

R1, R2, R4 and R5 independently represent hydrogen, amino carbonyl, C_{2-22}acyl, C_{1-22}alkyl which group is optionally substituted by one or two substituents selected from hydroxy and C_{1-22}alkoxy; C_{1-22} heteroalkyl, hydroxysulphonyl, C_{1-22}alkylsulphonyl, or C_{5-22}aryl sulphonyl;
R3 and R6 independently represent hydroxy; NH_2; C_{1-22}alkoxy wherein one or more carbons of the C_{1-22} alkyl may be optionally replaced by an aryl or an heteroaryl; C_{1-22} alkylamino which group is optionally substituted by a hydroxy or a C_{1-22} alkyl; hydroxy amino; C_{1-22} alkoxy amino; C_{1-22} alkylamino wherein one or more carbons of the C_{1-22}alkyl are replaced by an aryl or an heteroaryl; C_{1-22}heteroalkylamino; di(C_{1-22} alkyl)amino which is optionally substituted by a hydroxy; di(C_{1-22}alkyl)amino wherein one or more carbons of the C_{1-22}alkyl are replaced by an aryl or heteroaryl; or di(C_{1-22}heteroalkyl)amino;
R7 represents hydrogen, phosphate or sulphate;
L represents an optionally substituted C_{1-10}alkylene chain

with the proviso that the compound is not cysteine or cystine.

3. The method according to any one of claims 1 to 2, wherein the cysteine/cystine analogs are selected from the group consisting of N,N'-diacetyl-L-cystine-dimethylester, N-Acetyl-L-cysteine and N,N'-Diacetyl-L-cystine, or S-sulfo-cysteine.

4. The method according to any one of the preceding claims, wherein said cell culture medium comprises:

(a) cysteine/cystine analogs; and
(b) cysteine and/or cystine,

wherein the molar ratio of (a) to (b) is between 1:18 and 18:1.

5. The method according to any one of the preceding claims, wherein the method comprises the steps of:

(i) inoculating said host cells in a basal medium, wherein the basal medium optionally comprises an initial amount of:

(a) cysteine/cystine analogs; and/or
(b) cysteine and/or cystine,

(ii) progressing the culture through a production phase wherein the recombinant protein is produced by the cells, wherein, during said production phase, the cell culture medium is supplemented with:

(a) cysteine/cystine analogs; and/or
(b) cysteine and/or cystine,

wherein (a) and (b) may be added simultaneously or sequentially,
wherein, when the contents of the basal medium and the total supplements added are added up, the molar ratio of (a) to (b) is between 1:18 and 18:1.

6. The method according to claim 4 or 5, wherein said molar ratio of (a) to (b) is between 1:15 and 15:1, e.g. between

1:12 and 12:1, such as between 1:10 and 10:1, e.g. between 1:8 and 8:1, such as between 1:6 and 6:1, e.g. between 1:4 and 4:1, such as between 1:3 and 3:1, e.g. between 1:2 and 2:1, such as between 1.5:1 and 1:1.5.

7. The method according to claim 5 or 6, wherein the concentration of (b) in said basal medium is equivalent to between 0.05 and 5 mmol/L of cysteine, such as between 0.1 and 1 mmol/L, e.g. between 0.2 and 0.6 mmol/L.

8. The method according to any one of claims 5 to 7, wherein, during the production phase, the medium is supplemented with (a) cysteine/cystine analogs and (b) cysteine and/or cystine, wherein the sum of (a) and (b) added to the culture over the entire production phase is equivalent to between 1 and 75 mmol/L of cysteine, such as between 2 and 20 mmol/L.

9. The method according to any one of claims 5 to 8, wherein, during the production phase, the medium is supplemented daily with (a) cysteine/cystine analogs and (b) cysteine and/or cystine, wherein the daily addition brings the concentration of (a) + (b) to a concentration equivalent to between 0.05 and 5 mmol/L of cysteine, such as between 0.1 and 1 mmol/L.

10. The method according to any one of the preceding claims, wherein the host cells are mammalian cells, preferably CHO cells.

11. The method according to any one of the preceding claims, wherein the recombinant protein is an antibody or an antigen-binding fragment thereof.

12. The method according to any one of the preceding claims, wherein the antibody or antigen-binding fragment thereof is:

i) an antibody or antigen-binding fragment thereof which

a. comprises CDR-H1 having the sequence as defined in SEQ ID NO:1; CDR-H2 having the sequence as defined in SEQ ID NO:2; CDR-H3 having the sequence as defined in SEQ ID NO:3; CDR-L1 having the sequence as defined in SEQ ID NO:4; CDR-L2 having the sequence as defined in SEQ ID NO:5 and CDR-L3 having the sequence as defined in SEQ ID NO:6; or
b. comprises a light variable region having the sequence as defined in SEQ ID NO: 7 and a heavy variable region having the sequence as defined in SEQ ID NO: 8; or
c. comprises a light variable region having at least 80% identity or similarity, preferably 90% identity or similarity to the sequence as defined in SEQ ID NO: 7 and a heavy variable region having at least 80% identity or similarity, preferably 90% identity or similarity to the sequence as defined in SEQ ID NO: 8;
d. comprises a light variable region having the sequence as defined in SEQ ID NO: 7 and a heavy chain having the sequence as defined in SEQ ID NO: 11; or
e. comprises a light variable region having at least 80% identity or similarity, preferably 90% identity or similarity to the sequence as defined in SEQ ID NO: 7 and a heavy chain having at least 80% identity or similarity, preferably 90% identity or similarity to the sequence as defined in SEQ ID NO: 11; or

ii) an antibody which comprises a light chain having the sequence as defined in SEQ ID NO: 9 and a heavy chain having the sequence as defined in SEQ ID NO: 10; or
iii) an antibody which comprises a light chain having at least 80% identity or similarity, preferably 90% identity or similarity to the sequence as defined in SEQ ID NO: 9 and a heavy chain having at least 80% identity or similarity, preferably 90% identity or similarity to the sequence as defined in SEQ ID NO: 10.

13. The method according to any one of the preceding claims, wherein the recombinant protein is a protein which is not colorless at a concentration of 10 mg/ml or more, such as 50 mg/ml or more, when produced by host cells grown in a cell culture medium not comprising cysteine/cystine analogs.

14. A method for producing a recombinant protein preparation comprising:

(i) inoculating said host cells in a basal medium, wherein the basal medium optionally comprises an initial amount of:

(a) cysteine/cystine analogs as specified in claims 2 or 3; and/or
(b) cysteine and/or cystine,

(ii) progressing the culture through a production phase wherein the recombinant protein is produced by the cells, wherein, during said production phase, the cell culture medium is supplemented with:

(a) cysteine/cystine analogs; and/or
(b) cysteine and/or cystine,

wherein (a) and (b) may be added simultaneously or sequentially,
wherein, when the contents of the basal medium and the total supplements added are added up, the molar ratio of (a) to (b) is between 1:18 and 18:1.

15. A recombinant protein preparation obtainable or obtained by the method according to any one of the preceding claims.

16. A cell culture medium suitable for culturing mammalian cells comprising N,N'-diacetyl-L-cystine-dimethylester.

Figure 1 : Viable cell concentration

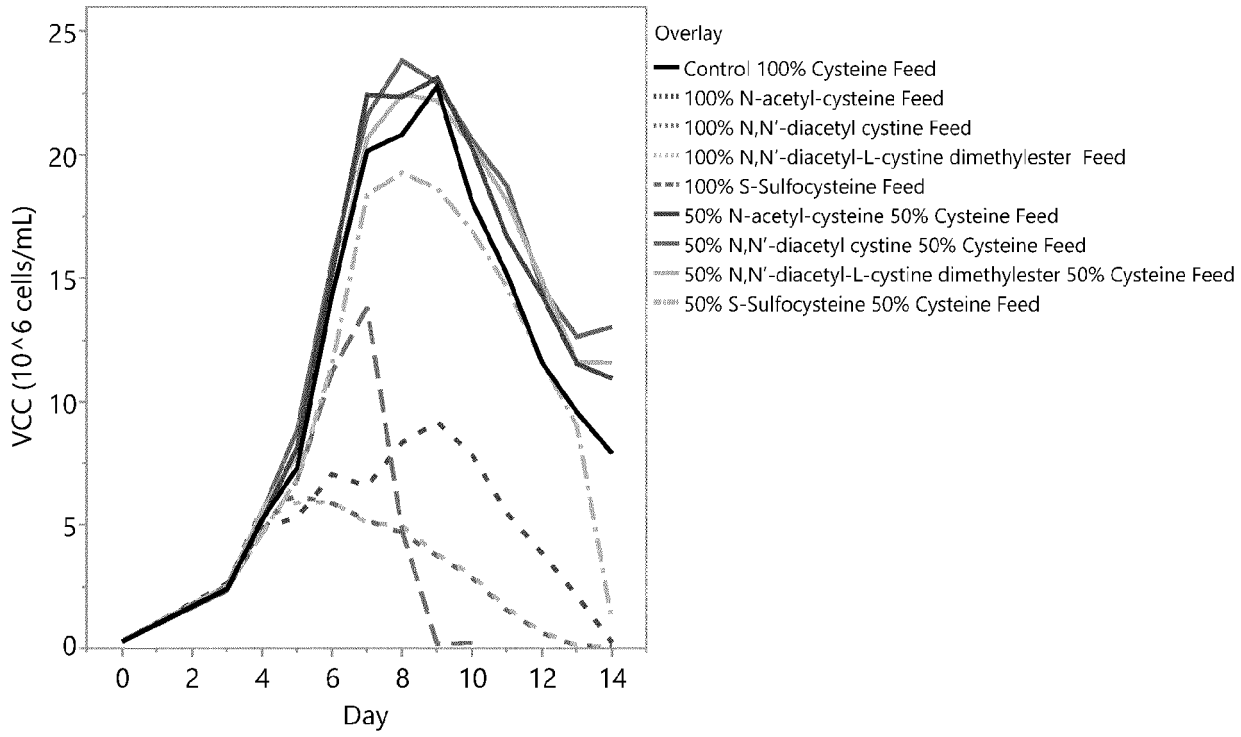


Figure 2 : Relative % change in Mab titer on day 14 with respect to control (100% Cysteine Feed)

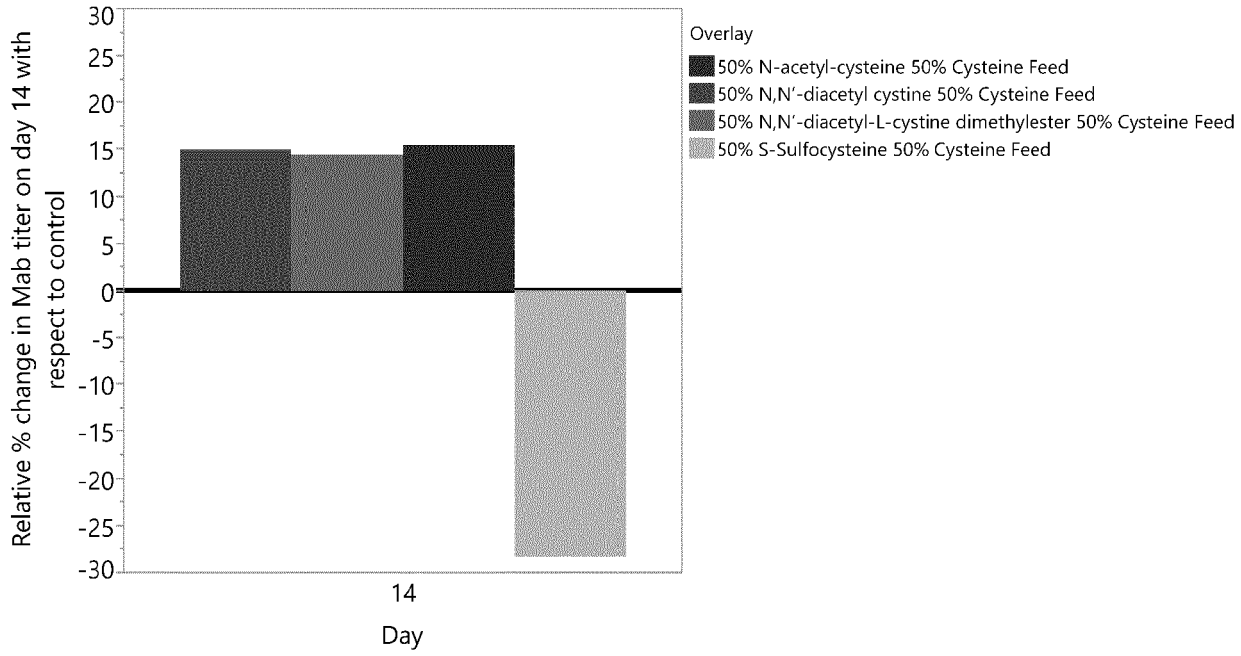


Figure 3 : Relative % change in color intensity (b*value) level on day 14 with respect to control (100% Cysteine Feed)

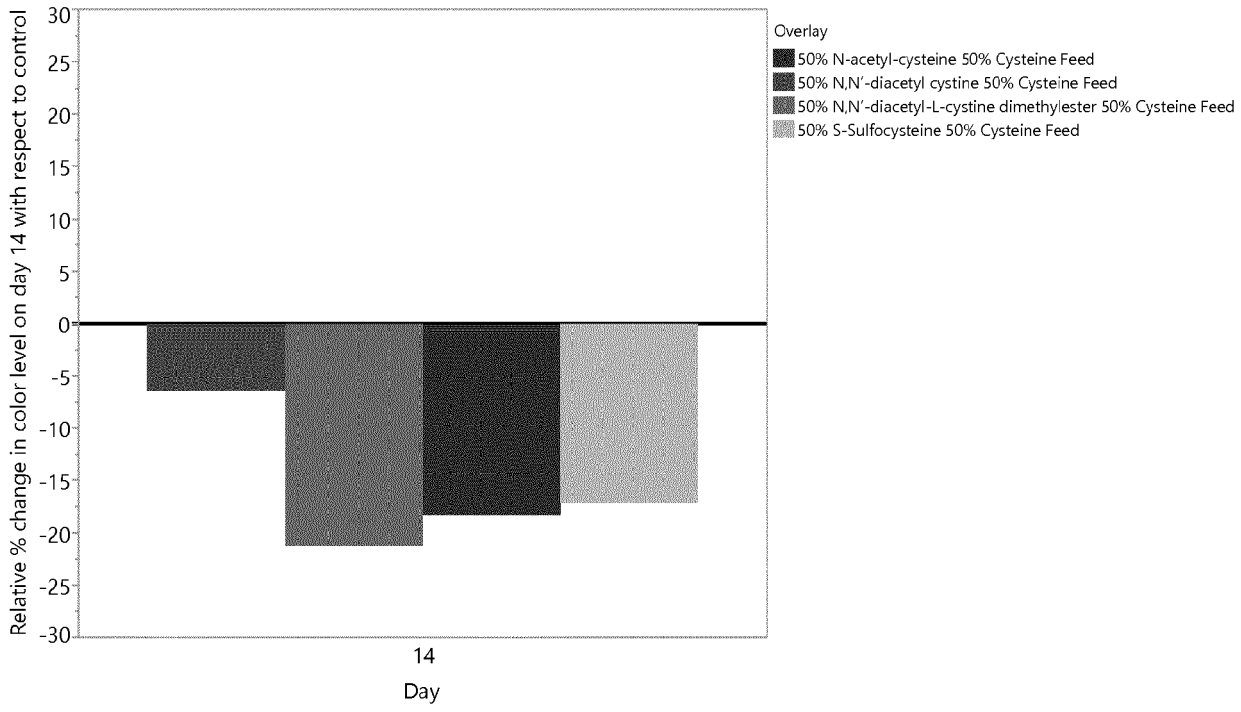


Figure 4 : Relative % change in acidic species level on day 14 with respect to control (100% Cysteine Feed)

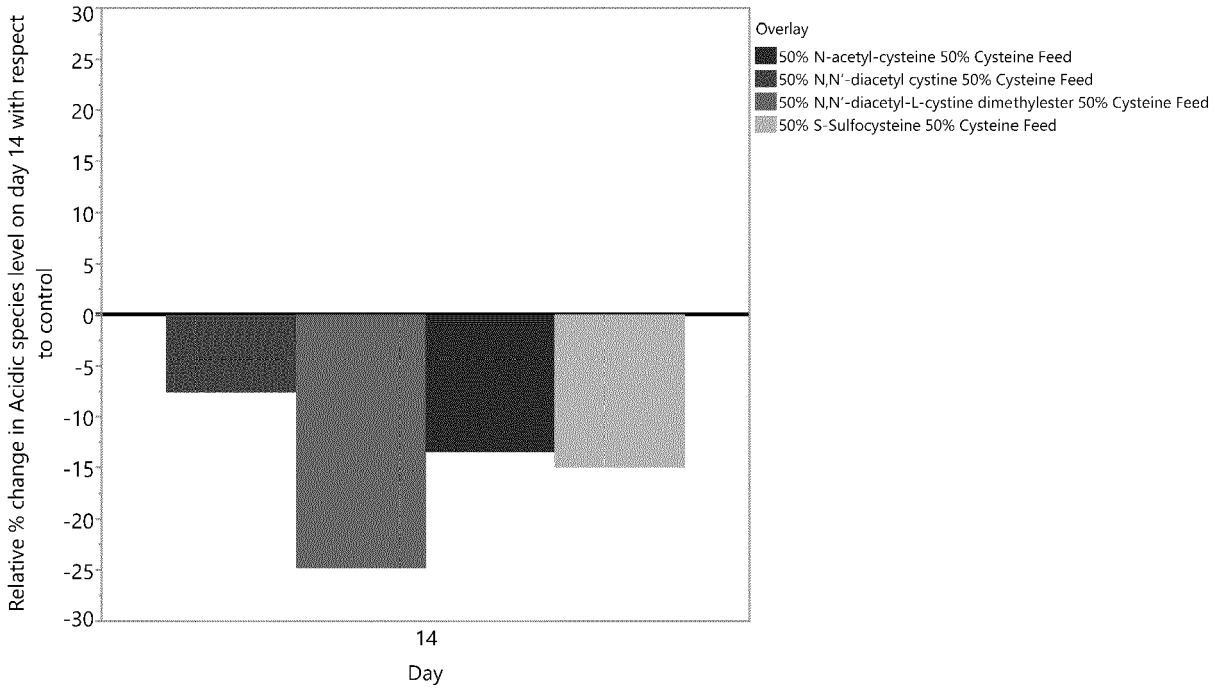


Figure 5 : Main charge species level on day 14

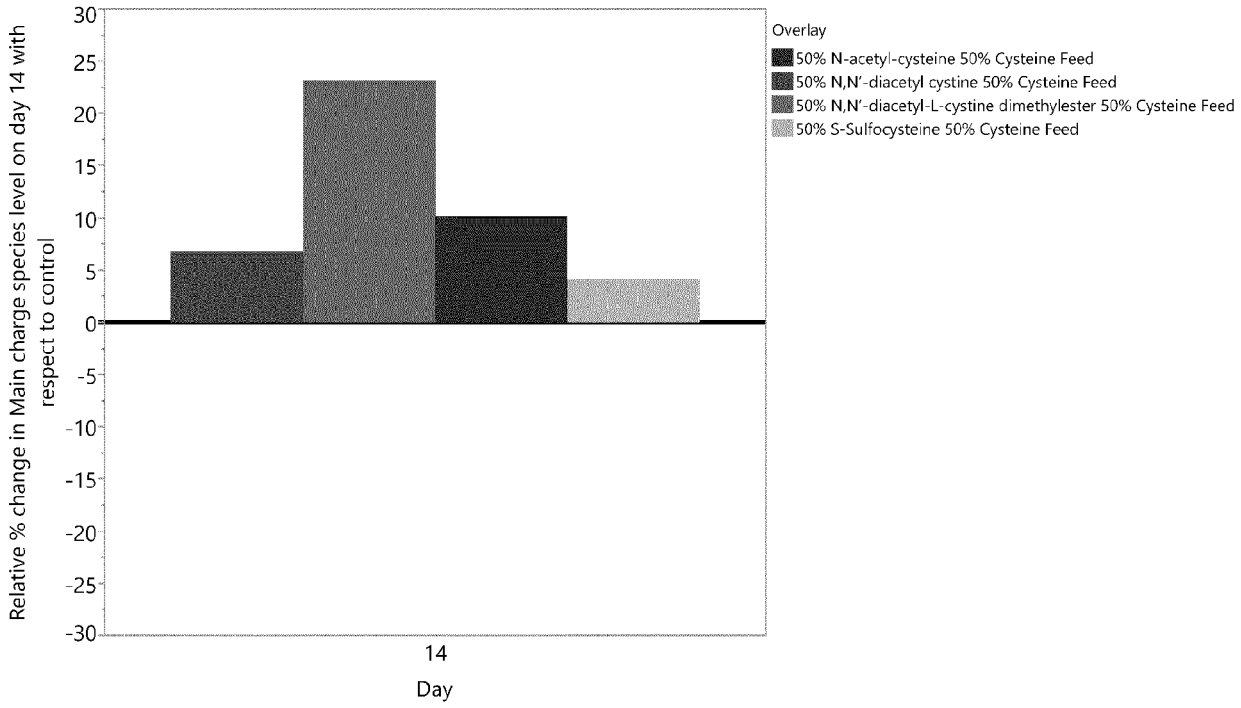


Figure 6 : Viable cell concentration

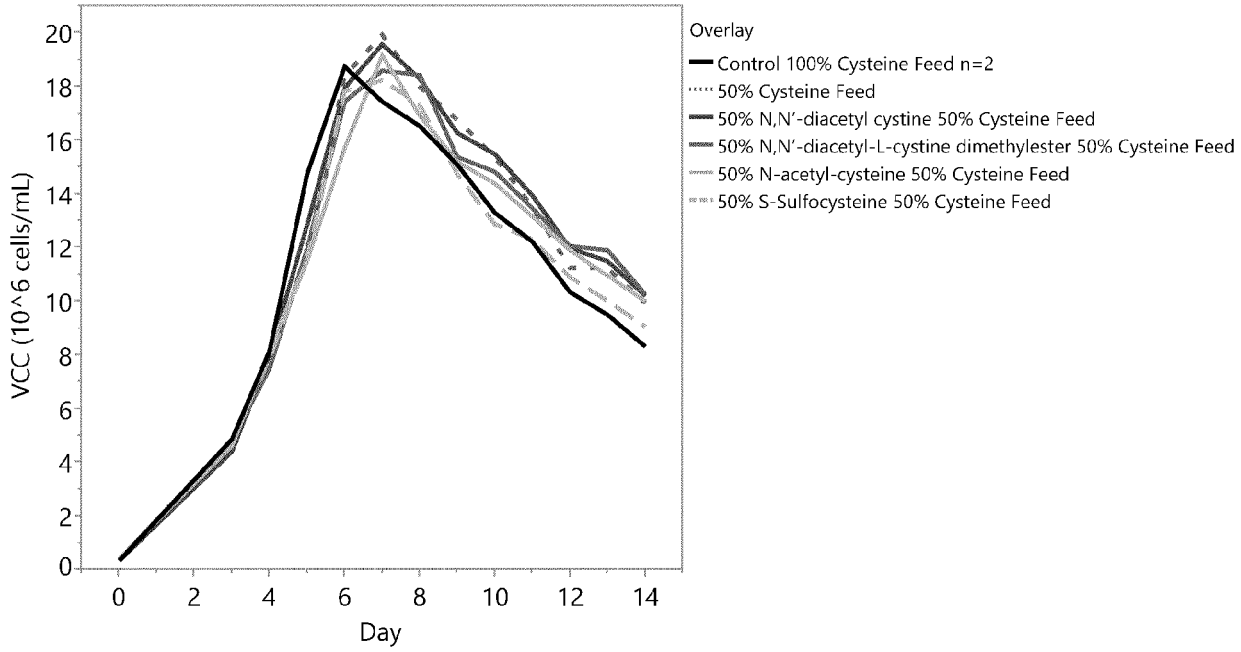


Figure 7 : Relative % change in Mab titer on day 14 with respect to control (100% Cysteine Feed)

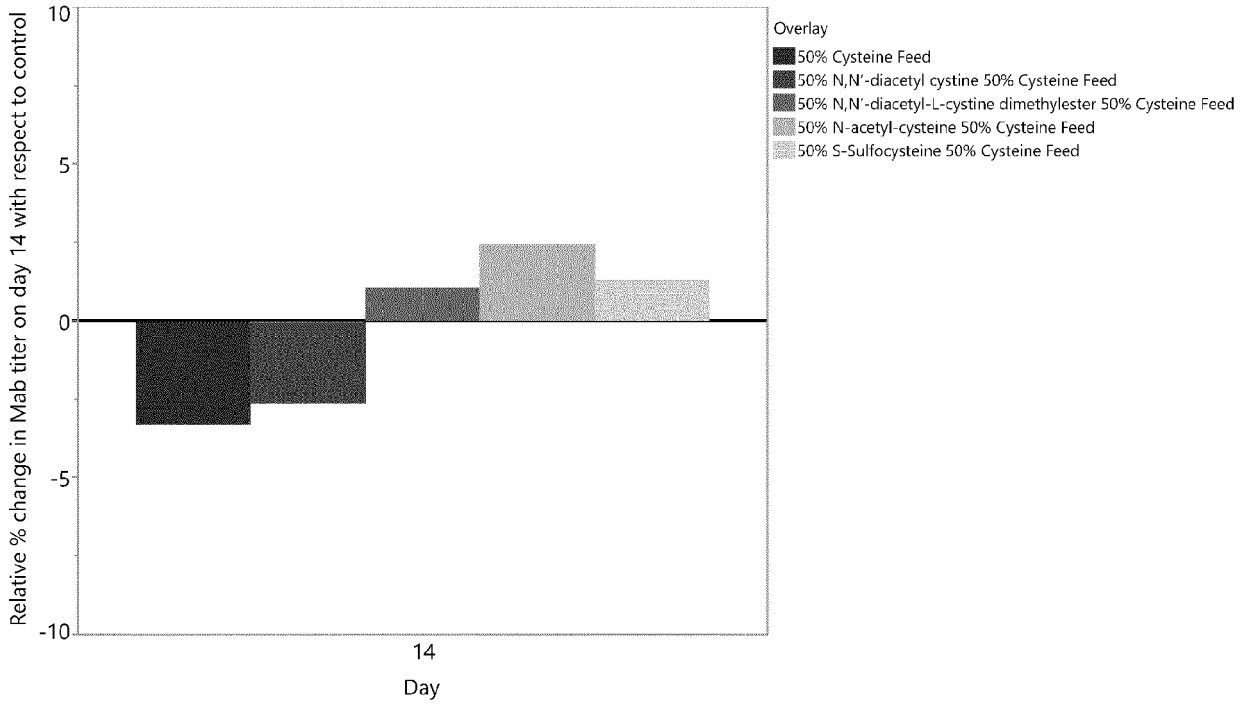


Figure 8 : Relative % change in acidic species level on day 14 with respect to control (100% Cysteine Feed)

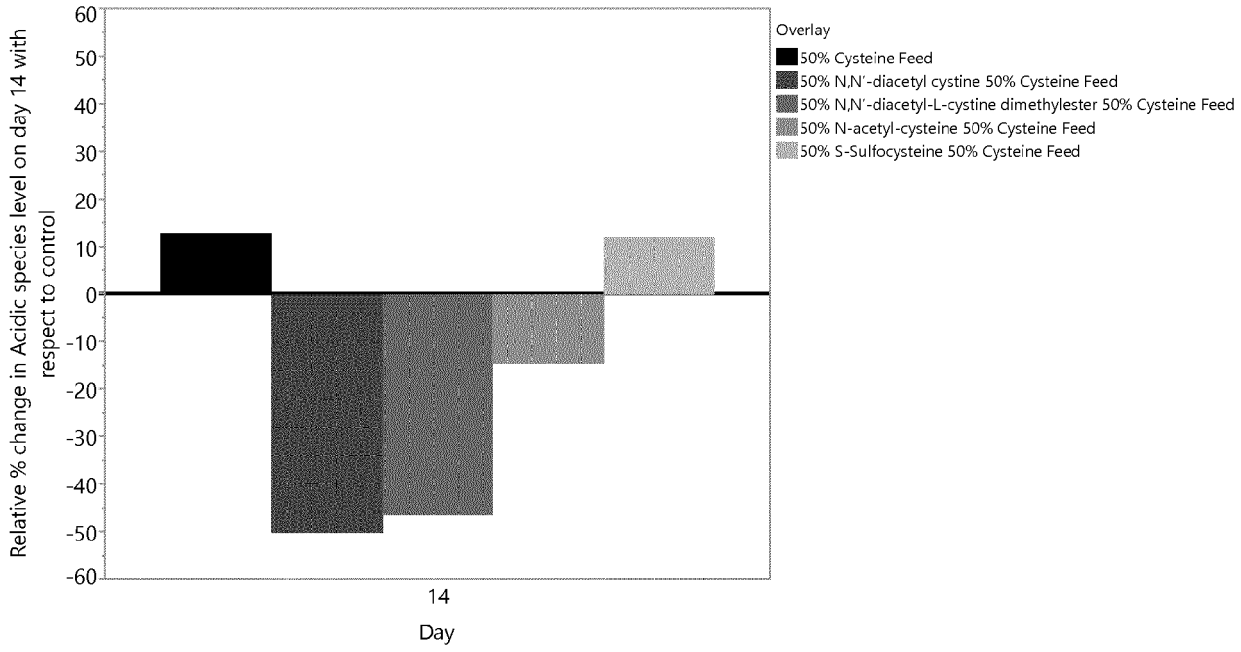


Figure 9 : Relative % change in main charge species level on day 14 with respect to control (100% Cysteine Feed)

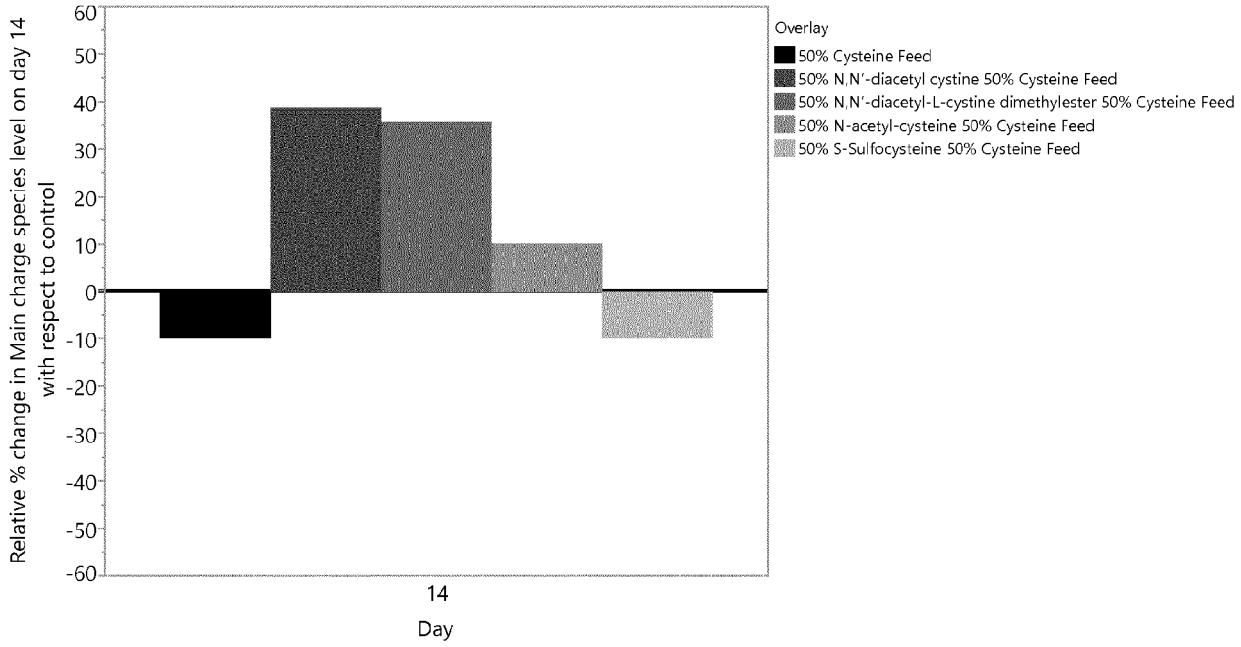


Figure 10 : Cell growth profile for cell line 1 in 2L bioreactor

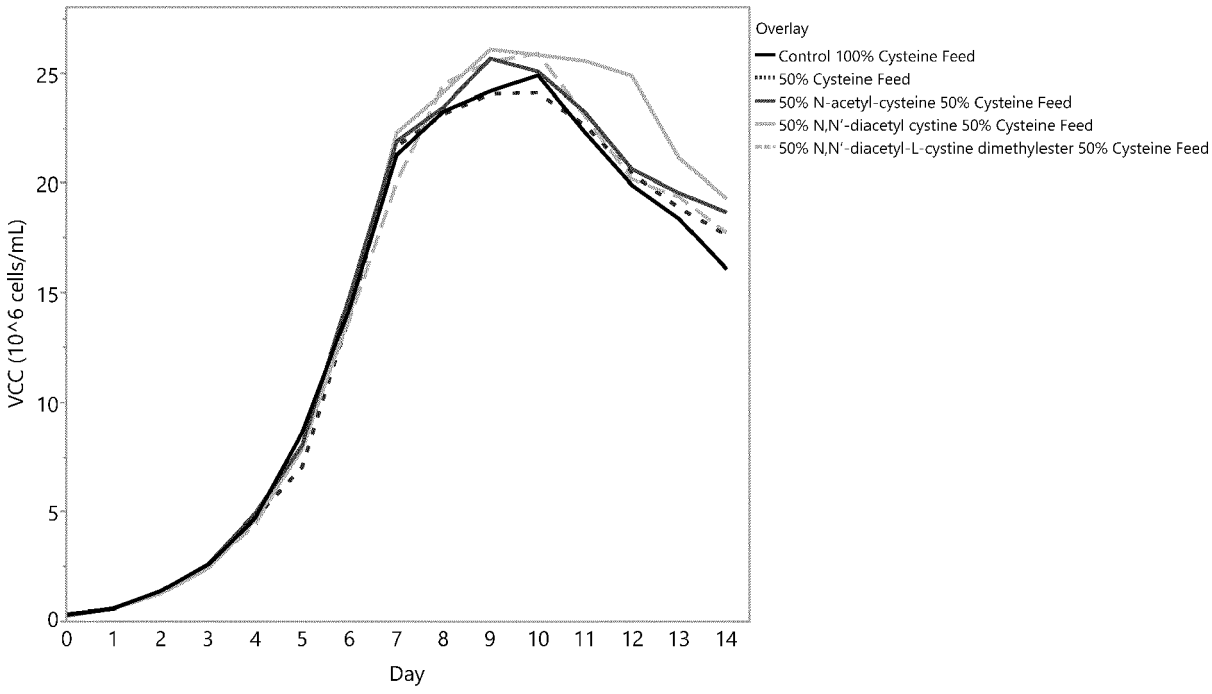


Figure 11 : Relative % change in Mab titer with respect to the control for cell line 1 in 2L Bioreactor

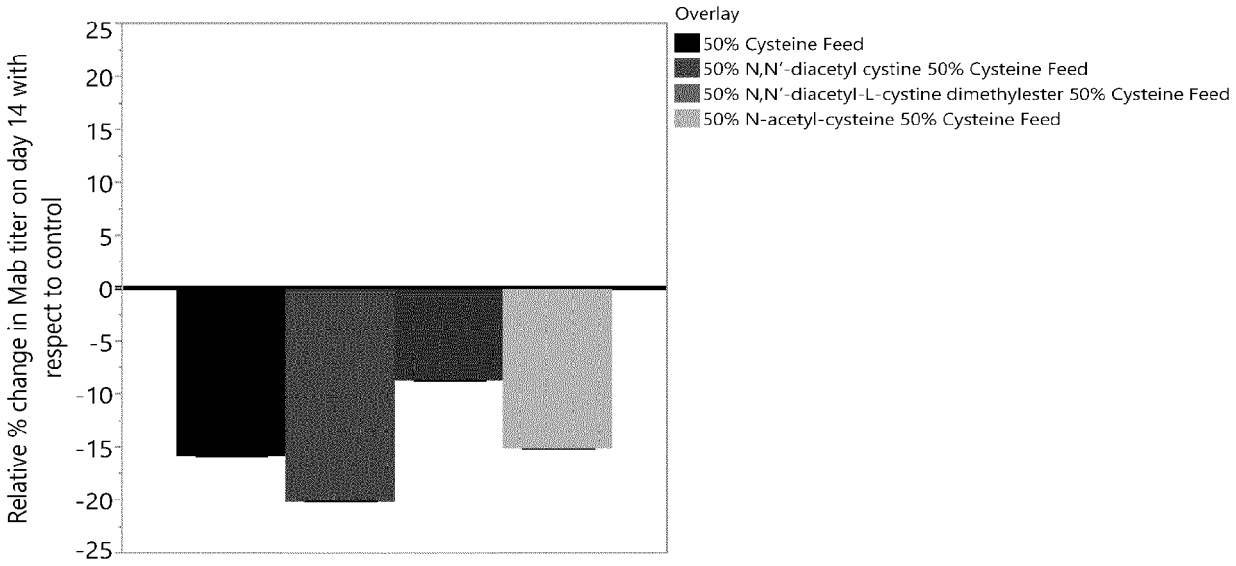


Figure 12 : Relative % change in acidic species level with respect to the control for cell line 1 in 2L bioreactor

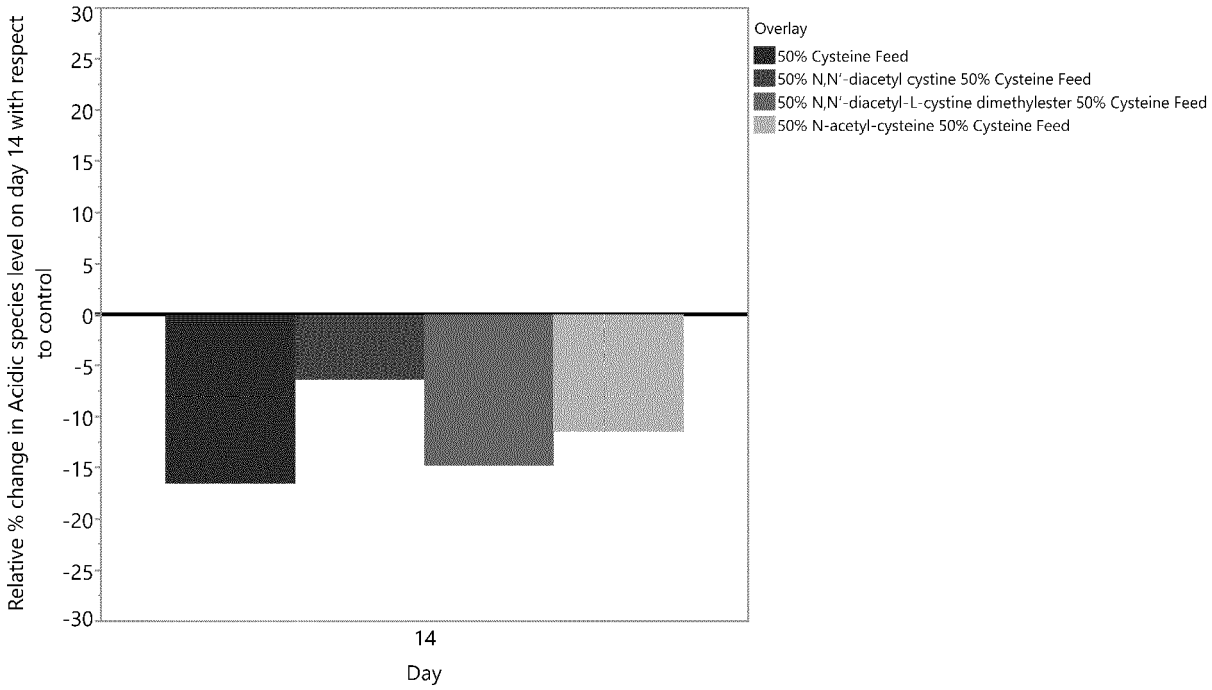


Figure 13 :Relative % change in main charge species level with respect to the control for cell line 1 in 2L bioreactor

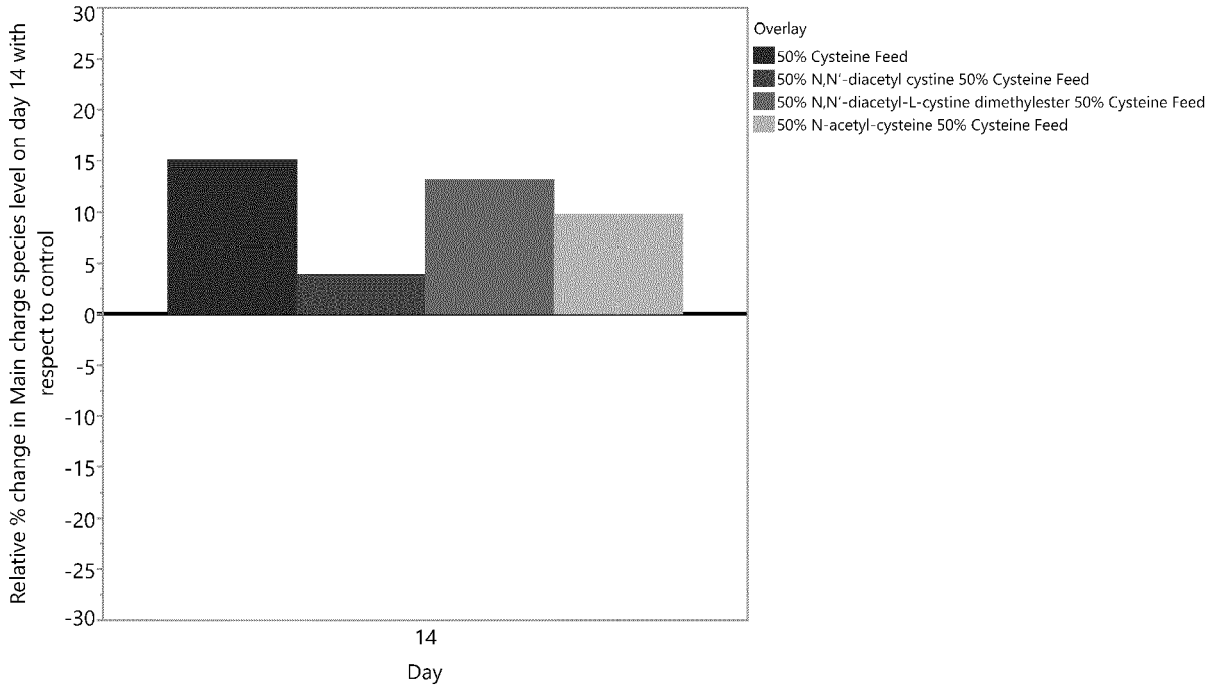


Figure 14 : Relative % change in color intensity (b* value) with respect to the control for cell line 1 in 2L bioreactor

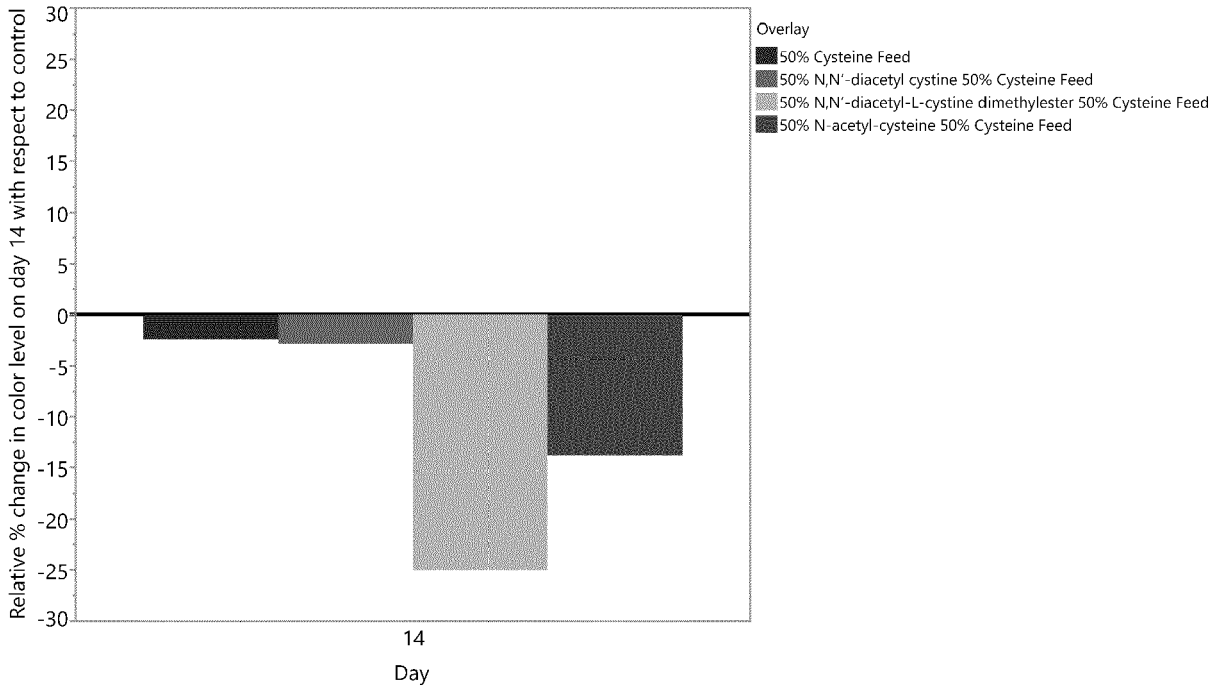


Figure 15 : Cell growth profile for cell line 2 in 2L bioreactor

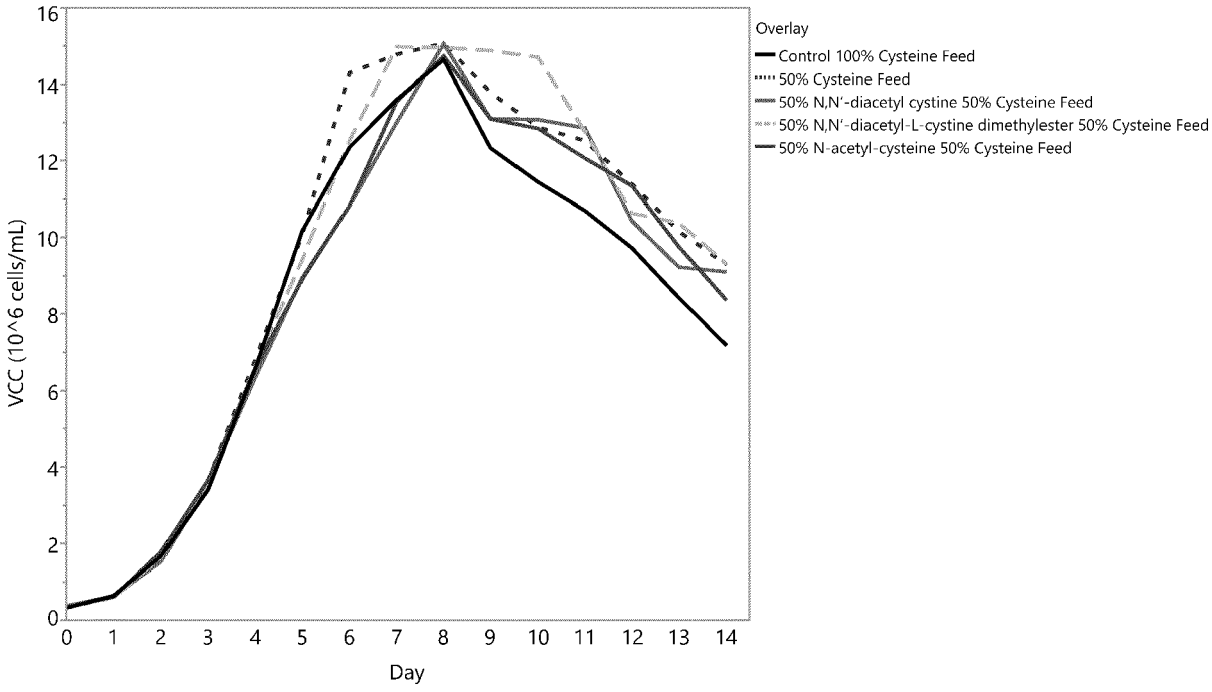


Figure 16 : Relative % change in Mab titer with respect to the control for cell line 2 in 2L Bioreactor

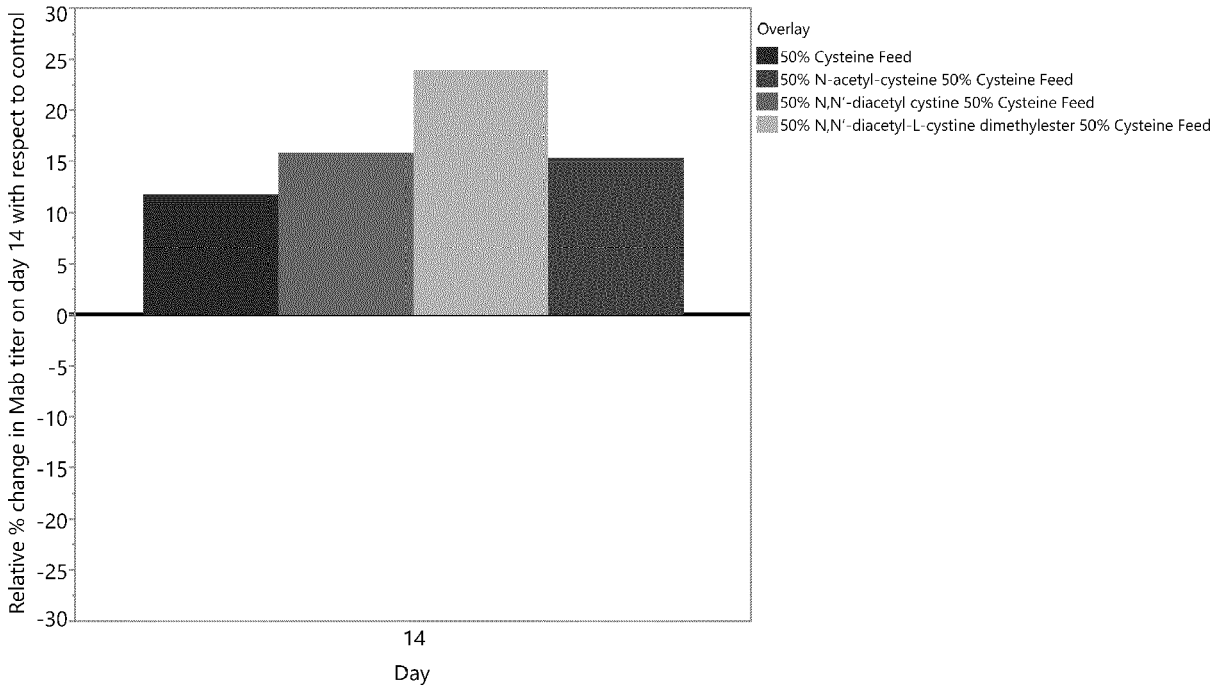


Figure 17 : Relative % change in acidic species level with respect to the control for cell line 2 in 2L bioreactor

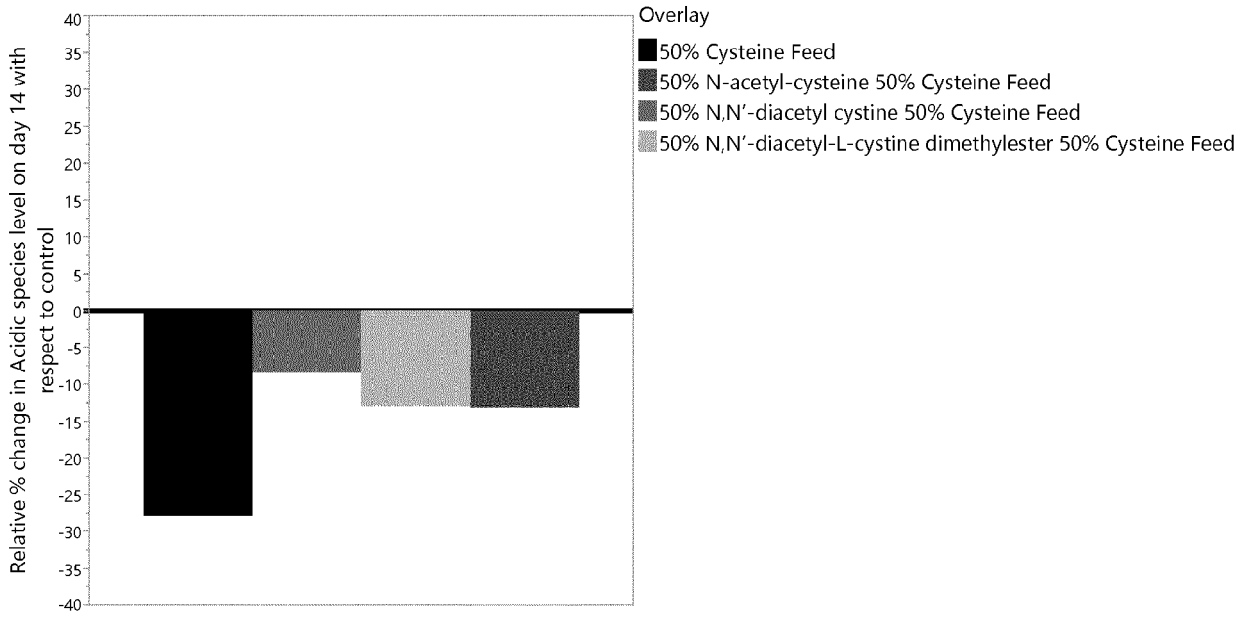


Figure 18 :Relative % change in main charge species level with respect to the control for cell line 2 in 2L bioreactor

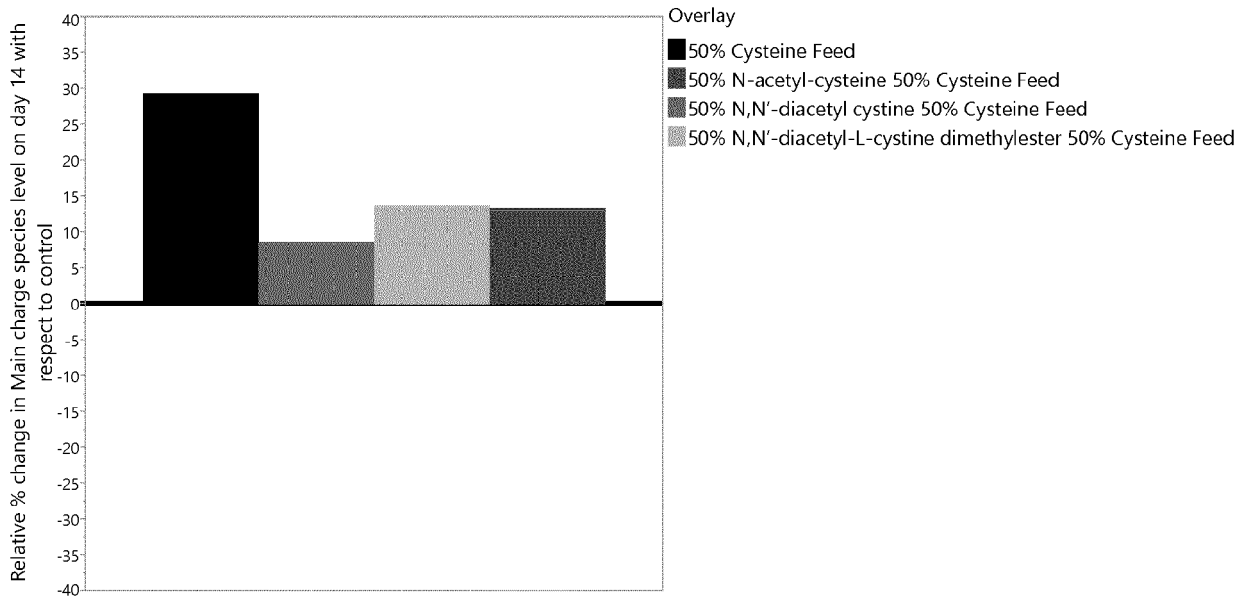


Figure 19 : Average cell growth profile from data sets 1 and 2 in 2L bioreactors (error bars = 1SD)

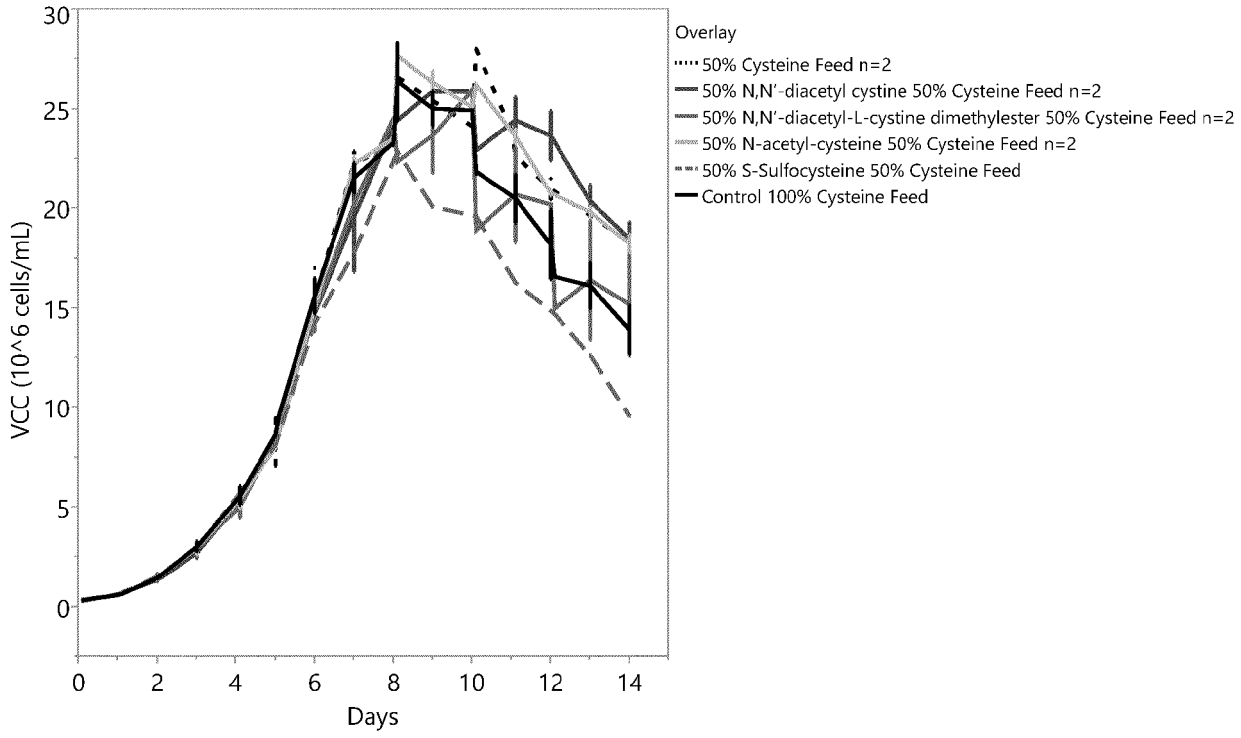


Figure 20 : Relative % change in Mab titer with respect to the control. Data sets 1 and 2 average values (error bars = 1SD)

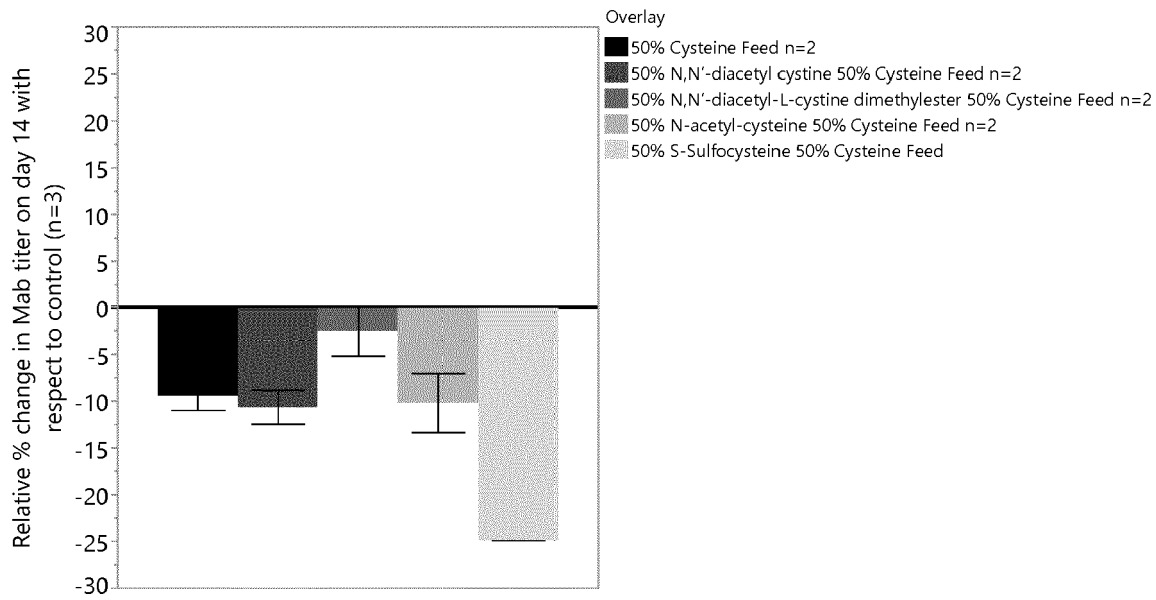


Figure 21 : Relative % change in acidic species level with respect to the control. Data sets 1 and 2 average values (error bars = 1SD)

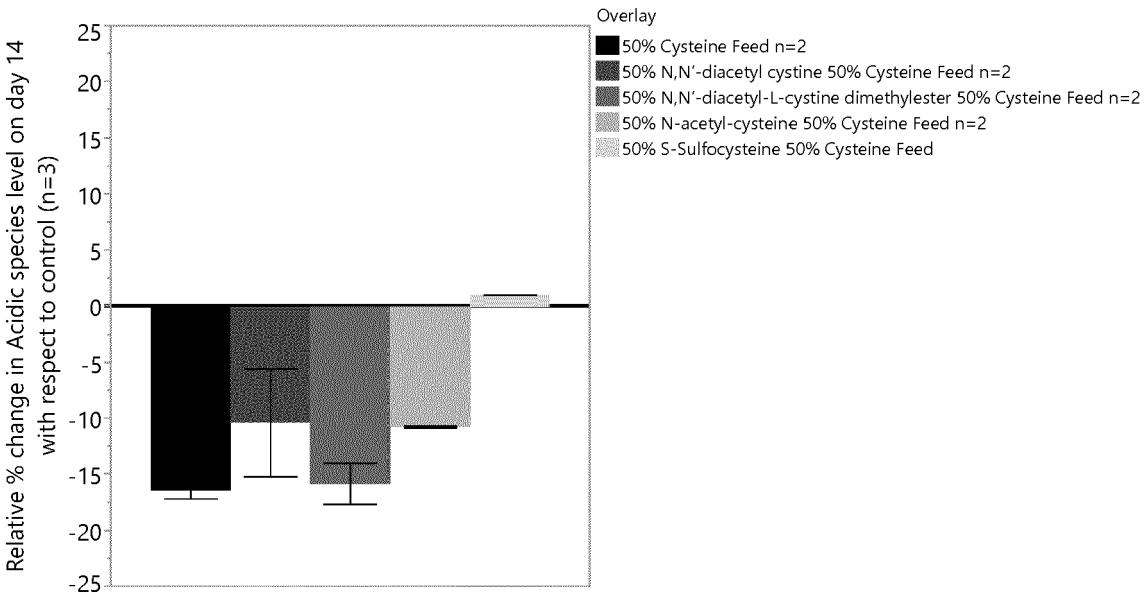


Figure 22 : Relative % change in main charge species level with respect to the control. Data sets 1 and 2 average values (error bars = 1SD)

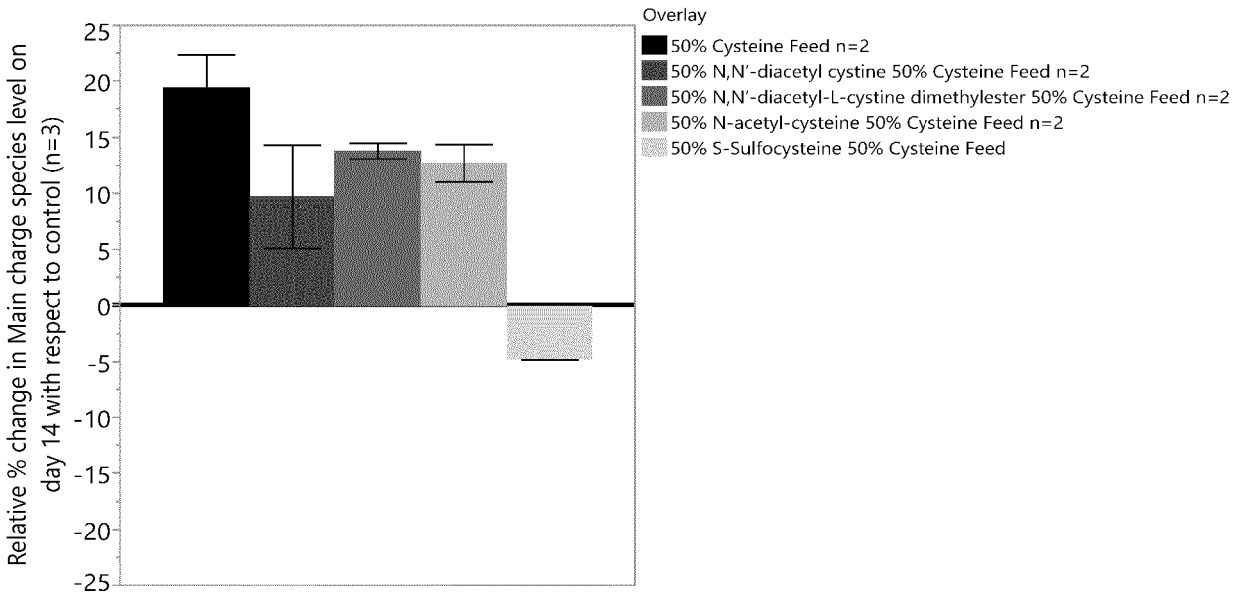
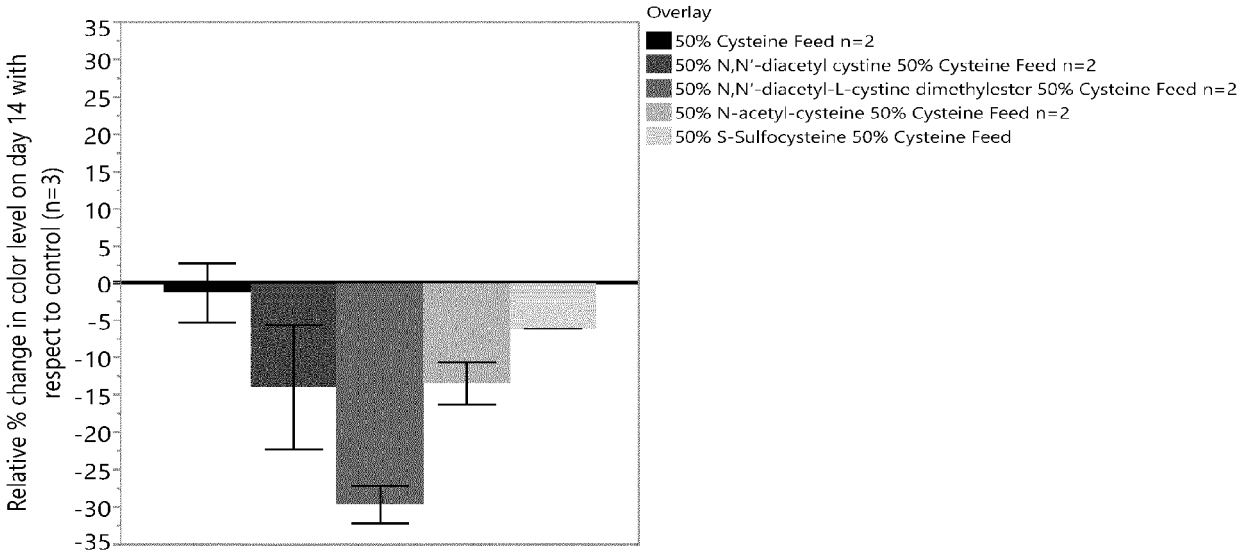


Figure 23 : Relative % change in color intensity (b* value) with respect to the control. Data sets 1 and 2 average values (error bars = 1SD)



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EUROPEAN SEARCH REPORT

Application Number
EP 17 20 4978

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (IPC)
X Y	WO 2017/186654 A1 (MERCK PATENT GMBH [DE]) 2 November 2017 (2017-11-02) * page 9, paragraph; claims 1-11 *	1-5,10, 11,13-15 1-16	INV. C12N5/00
X	RONJA SEIBEL ET AL: "Impact of S-sulfocysteine on fragments and trisulfide bond linkages in monoclonal antibodies", MABS, vol. 9, no. 6, 5 June 2017 (2017-06-05), pages 889-897, XP055465718, US ISSN: 1942-0862, DOI: 10.1080/19420862.2017.1333212	1-5,10, 11,13-15	
Y	* the whole document *	1-16	
X	HAN KYU OH ET AL: "Effect of N-Acetylcystein on Butyrate-Treated Chinese Hamster Ovary Cells To Improve the Production of Recombinant Human Interferon-&bgr;-1a", BIOTECHNOLOGY PROGRESS, vol. 21, no. 4, 1 January 2005 (2005-01-01), pages 1154-1164, XP055034659, ISSN: 8756-7938, DOI: 10.1021/bp050057v	1-5,10, 13-15	
Y	* the whole document *	1-16	
	-/--		

TECHNICAL FIELDS SEARCHED (IPC)
C12N

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
Munich	10 April 2018	Offermann, Stefanie

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
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A : technological background
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D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

EPO FORM 1503 03.82 (P04C01)

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EUROPEAN SEARCH REPORT

Application Number
EP 17 20 4978

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (IPC)
X	HECKLAU CAROLINE ET AL: "S-Sulfocysteine simplifies fed-batch processes and increases the CHO specific productivity via anti-oxidant activity", JOURNAL OF BIOTECHNOLOGY, ELSEVIER, AMSTERDAM, NL, vol. 218, 2 December 2015 (2015-12-02), pages 53-63, XP029379422, ISSN: 0168-1656, DOI: 10.1016/J.JBIOTEC.2015.11.022	1-5,10, 11,13-15	
Y	* the whole document * * figure 2 *	1-16	

X	WO 2008/033517 A2 (ABBOTT LAB [US]; PLA ITZCOATL A [US]; MATUCK JOSEPH C [US]; FANN JOHN) 20 March 2008 (2008-03-20)	1-5,10, 11,13-15	
Y	* page 17, paragraph 2; claim 214 *	1-16	

X	KITAZAWA M ET AL: "Intracellular redox regulation by a cystine derivative suppresses UV-induced NF-@kB activation", FEBS LETT, ELSEVIER, AMSTERDAM, NL, vol. 526, no. 1-3, 28 August 2002 (2002-08-28), pages 106-110, XP004379234, ISSN: 0014-5793, DOI: 10.1016/S0014-5793(02)03152-6 * the whole document *	16	TECHNICAL FIELDS SEARCHED (IPC)

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
Munich	10 April 2018	Offermann, Stefanie

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
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D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

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ANNEX TO THE EUROPEAN SEARCH REPORT ON EUROPEAN PATENT APPLICATION NO. EP 17 20 4978

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10-04-2018

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